DOCTOR OF PHILOSOPHY

Investigation of plant derived compounds mediating cell signalling effectson FOXO1a and AMPK

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Award date:
2012

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University of Dundee

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By

Sandra Bacon

A thesis presented to the University of Dundee for the degree of Doctor of Philosophy

September 2012

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Acknowledgements

I would like to take some time to thank those who have supported me during my PhD research. I have a particular debt of gratitude to my supervisors Dr Graham Rena and Dr Gordon McDougall whose knowledge and enthusiasm served as a source of inspiration and motivation.

Thanks also to Dr Amy Cameron, Dr Jean Harthill and Lisa Logie from Dundee University Medical Schoolfor technical help and advice and also to Dr Sean Conner, Pat Dobson and Johannes Rauscher from the James Hutton Institute.

I have further to thank Mohammed Al-Khairulla and Paul Middleton, the honours year students who helped produce some of this data.

Finally I would like to thank my friends and family who have unwaveringly supported me throughout.

Candidate's Declaration

I hereby declare that all results described in this thesis, unless otherwise stated, are entirely my own work. I further state that the composition of this thesis was performed by myself and none of the material has been submitted for any other degree. Lastly, I verify that all sources have been appropriately cited. This work was carried out in the James Hutton Institute, Invergowrie, Dundee under the supervision of Dr Gordon McDougall and the Centre for Neuroscience (now Division of Cardiovascular and Diabetes Medicine) under the supervision of Dr. G. Rena.

Sandra Bacon

Supervisor's Declaration

I certify that Sandra Bacon has completed nine terms of experimental research and has fulfilled the conditions of Ordinance 39, University of Dundee, such that she is eligible to submit the following thesis in application for the degree of Doctor of Philosophy.

Dr. Graham Rena

Summary

In recent years there has been a considerable increase in human obesity levels with an associated increase in the incidence of type 2 diabetes mellitus (T2DM), a disorder of glucose metabolism characterised by insulin resistance. It is unclear why obesity and insulin resistance should frequently exist comorbidly but the current prevalence of polypharmacy suggests the underlying mechanisms may be multifactorial in origin. This project has highlighted plant compounds and extracts with:

Insulin-like properties in cell culture experiments in that they induce phosphorylation and therefore inactivation of the transcription factor FOXO1a, which is a major downstream effector insulin and

Properties similar to the T2D drugs metformin and pioglitazone, which both activate AMOK signalling and reduce phosphorylation of the ribosomal protein S6.

The project began with an analysis of plant extracts which can mediate intracellular cell signalling effects on FOXO1a and AMPK. The pilot data established that one extract (grape seed, GSE) induces regulation of AMPK and FOXO1a much more readily than another (pine bark, PBE). GSE and PBE were subjected to multiple fractionation methods and mass spectrometry to learn more about the active agent(s) in the extracts. Another chapter adopted a candidate-approach, investigating effects of the plant compound gallic acid (GA) on AMPK. Although GA cannot explain the effects of GSE on AMPK, the availability of a variety of analogues of GA allowed investigation of structural requirements for cell responses. Two more extracts, cranberry and lingonberry, were then investigated using the

techniques established earlier with GSE and PBE. These studies discovered that fractions containing B-type linkages were more effective at phosphorylating of FOXO1a than those containing A-type linkages, suggesting B-type linkages may be required for these effects.
Metformin itself is a synthetic analogue of a plant compound and metformin analogues known as diguanides were synthesised and structure/activity relationships were assessed. Diguanides and biguanides were found ti induce similar responses but diguanides were much more toxic than biguanides, suggesting they may be less specific in their mechanism of action, or alternatively that they have different intramitochondrial targets.

The aims of this project were

1. To extract polyphenolic compounds from grape seed, pine bark and berries using chromatography techniques and to characterise these compound using liquid chromatography/mass spectrometry
2. To exclude or confirm insulin-like or metformin-like properties by application to cell culture models to assess effects on regulation of glucose and energy homeostasis by measuring phosphorylation of the transcription factor FOXO1a, AMPK and the ribosomal protein S6.

Abbreviations

ACC	acetyl CoA carboxylase
ACE	angiotensin converting enzyme
ACN	acetonitrile
AD	Alzheimer's Disease
ADP	adenosine diphosphate
AG	alkyl guanidine
AICAR	5-aminoimidazole-4-carboxamide riboside
AMP	adenosine monophosphate
AMPK	5' AMP-activated protein kinase
AMU	atomic mass units
APP	amyloid precursor protein
ASP	aspirin
ATP	adenosine triphosphate
Aβ	amyloid-β peptide
BA	benzoic acid
BTE	black tea extract
CamKI	calcium/calmodulin-dependent protein kinase 1
cAMP	cyclic adenosine monophosphate
CB	cranberry
CBS	cystathionine β-synthase
CDC	Centers for Disease Control and Prevention
CK1	casein kinase-1
CNS	central nervous system
DAF	dauer arrest phenotype
DAG	diacylglycerol
DG	diguanide
DMEM	Dulbecco's modified eagle's medium
DNP	2,4 dinitrophenol
ECL	enhanced chemoluminescence
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ES	embryonic stem
FA	formic acid
FBS	fetal bovine serum
FFA	free fatty acids
FKBP	FK506 binding protein
FOX	Forkhead box
FRAP	ferrous reducing antioxidant power
FSD	full scale deflection
GA	gallic acid
GAE	gallic acid equivalent
G6Pase	glucose-6-phosphatase
GFP	green fluorescent protein
GLUT4	glucose transporter 4
GSE	grape seed extract
GSK3	glycogen synthase 3
H_2O_2	hydrogen peroxide
HBA	hydroxy benzoic acid

HDL	high density lipoprotein
HEK	human embryonic kidney
HMGR	3-OH-3-methylglutaryl CoA reductase
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
HSL	hormone sensitive lipase
ICAM1	Intracellular adhesion molecule 1
IIS	insulin/IGF-1 signalling
IGF	insulin-like growth factor
IGF-1R	IGF-1 receptor
IkB	inhibitory κβ
IKK	inhibitory κ kinase
IL8	interleukin 8
IR	insulin receptor
IRS	insulin receptor substrate
JNK	c-jun N-terminal kinase
KO	knockout
LB	lingonberry
LC/MS	liquid chromatography/ mass spectrometry
LDL	low density lipoprotein
MALDI	matrix assisted laser depolarisation/ionisation
MAPK	mitogen-activated protein kinase
MCP1	monocyte chemotactic protein
MIRKO	muscle specific insulin receptor knock out
MOPS	morpholino propane sulphonic acid
mTOR	mammalian target of rapamycin
mTORC	mammalian target of rapamycin complex
MW	molecular weight
M/Z	mass to charge ratio
NADP	nicotinamide adenine dinucleotide phosphate
NES	nuclear exclusion sequence
NF–kB	nuclear factor κ light chain enhancer of activated B cells
NLS	nuclear localisation sequence
NOS	nitric oxide synthase
O_2^-	superoxide
OCT1	organic cation transporter 1
$p70^{RSK}$	p70 ribosomal protein S6 kinase
p85	85 kDa regulatory
$p90^{RSK}$	MAPK-activated protein kinase-1
PAC	proanthocyanidin
PBE	pine bark extract
PDK1	3-phosphoinositide-dependent kinase 1
PEPCK	phosphoenolpyruvate carboxykinase
PH	pleckstrin homology
PI	phosphatidylinositol
PIF	PDK-1 interacting fragment
PIP2	phosphoinositide biphosphate
PIP3	Phosphoinositide triphosphate
PI3-kinase	phosphoinositide 3-kinase

PKB	protein kinase B/akt
PKC	protein kinase C
PMSF	phenyl methane sulphonyl fluoride
PPAR	peroxisome proliferator activated receptor
PTB	phosphotyrosine binding
RT	retention time
S6	p70 ribosomal protein S6
S6K	p70 ribosomal protein S6 kinase
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH2	src homology 2
SIGN	Scottish Intercollegiate Guidelines Network
SOD	superoxide dismutase
SPE	solid phase extraction
T1D	type 1 diabetes mellitus
T2D	type 2 diabetes mellitus
TBS	tris buffered saline
TBST	tris buffered saline with tween
TG	triglyceride
TNFα	tumour necrosis factor α
TSC	tuberous sclerosis complex (protein)
TZDs	thiazolidinediones
UPW	ultra pure water
WHO	World Health Organisation
WST1	water soluble tetrazolium salt 1
ZDF	Zucker diabetic fatty (rat)
ZMP	AICAR monophosphate

CHAPTER 1 – INTRODUCTION

1.1 Diabetes

Diabetes mellitus is a group of metabolic disorders characterised by hyperglycemia either because the patient does not produce enough insulin or because somatic cells are resistant to it (Greenspan *et al*, 2011).

In 1500BC, a condition was described in Egypt and India with these symptoms

- excessive weight loss
- extreme thirst
- “too great an emptying of the bladder” (polyuria)
- “honey urine” (glycosuria) and
- ketosis

This condition was called “diabetes” meaning “siphon” or “to pass through”.

In 1776, the sweet tasting urine and serum of diabetic patients was confirmed to be due to sugar (Zajac *et al*, 2010). In 1788, a link between diabetes and the pancreas was suggested when it was noted that pancreatic pathology could result in diabetes and this was later confirmed by the discovery of insulin secretion from the pancreas by Banting and Best (Zajac *et al*, 2010).

1.1.1Epidemiology

The World Health Organisation (WHO) state that 347 million people suffer from diabetes world wide. In 2004 they estimated 3.4 million people died from the consequences of hyperglycemia. In 2012 they projected the number

of diabetes deaths will increase by two thirds between the years 2004 and 2012 (WHO, 2012).

1.2 Glucose

Glucose is a monosaccharide used as a source of energy in most organisms (De Fronzo *et al*, 2004). In humans, it originates from dietary polysaccharides, mainly starch, and is stored predominately in the liver by insulin-controlled pathways as polymeric glycogen (De Fronzo *et al*, 2004). When blood glucose levels are low, the hypoglycaemic state, α-cells of the endocrine pancreas secrete the hormone glucagon which induces hepatic catabolism of glycogen to glucose (De Fronzo *et al*, 2004). In the *post prandial* state some glucose enters the liver via the hepatic portal vein and meanwhile circulating glucose stimulates pancreatic β cells to release insulin which promotes hepatocyte uptake of glucose and storage as glycogen (De Fronzo *et al*, 2004). It has also been proposed that when hepatic glucose levels exceed arterial glucose levels a 'portal signal' increases hepatic glucose uptake independently of the insulin response (Flakoll *et al*, 2004). Glucose disposal into adipocytes and muscle is also significant but these cells are not studied in this thesis.

Plasma glucose levels are tightly regulated in healthy individuals. Normal fasting plasma glucose levels are 4 - 5 mmol/l (American Diabetes Association, 2006). Levels between 5.6 mmol/L and 7.0 mmolL are considered higher than normal range and are referred to as "pre-diabetes" (American Diabetes Association, 2006). Levels of 7mmol/L or higher are indicative of diabetes (American Diabetes Association, 2006).

1.3 Insulin

The pancreas is an organ which consists of an exocrine portion which secretes digestive enzymes into the small intestine and an endocrine portion of pancreatic islets embedded within the exocrine pancreas containing α- and β-cells which secrete glucagon and insulin respectively (De Fronzo *et al*, 2004). Insulin is an evolutionarily conserved hormone synthesised from a single chain precursor, preproinsulin, which consists of four domains, a signal peptide and an A and a B domain linked by a C domain (Rhodes, 2004). In pancreatic β-cells, preproinsulin undergoes two proteolytic cleavage events involving removal of the signal peptide and the C domain accompanied by specific pairing of disulphide bridges. These events result in the production of mature insulin, which is a globular protein consisting of only two of the original chains, A and B, of 21 and 30 residues respectively, linked together by two disulphide bonds (Weiss, 2009).

1.4 Insulin receptor

The insulin receptor (IR) gene expresses two isoforms generated by alternative splicing (LeRoith, 2003). The isoforms differ in that they lack (IR-A) or contain (IR-B) a 12 amino acid peptide encoded by exon 11 of the IR gene (LeRoith, 2003). Stimulation of the tyrosine kinase activity of the insulin receptor (IR) by insulin initiates a phosphorylation cascade that mediates the pleiotropic actions of this hormone (Avruch, 2004). *In vivo* studies have confirmed insulin receptor physiology to be tissue specific (Almind *et al*, 2004; Kadowaki , 2000). Global IR knockout (KO) mice have a severe diabetic phenotype which is fatal within one week postnatally (Almind *et al*,

2004; Kadowaki, 2000). Muscle specific IR KO (MIRKO) mice show defective fat metabolism which leads to development of metabolic syndrome (Almind *et al*, 2004; Kadowaki, 2000). In humans, metabolic syndrome is defined as the presence of central obesity and two other factors from hypertension, hyperglycemia, raised triglyceride (TG) levels and reduced high density lipoprotein (HDL) levels (International Diabetes Federation, 2006). MIRKO mice displayed increased fat mass, elevated TG and free fatty acid (FFA) levels but no hyperglycemia (Almind *et al*, 2004; Kadowaki, 2000). Although hepatic IR KO mice show severe insulin resistance and glucose intolerance by the age of two months, by four months of age, fasting hyperglycemia returns to normal (Almind *et al*, 2004; Kadowaki, 2000), suggesting that the presence of intact insulin receptors in other tissues may compensate for the knockout.

1.5 Insulin- like growth factor

Insulin-like growth factors (IGFs) are polypeptides with high protein sequence homology to insulin (Ullrich *et al*, 1986). The IGF system includes two ligands, IGF1 and IGF2 and two cell surface receptors, IGF1R and IGF2R (Ullrich *et al*, 1986). IGF1 is a single chain polypeptide of 70 residues with a significant 49% structural sequence identity to insulin and 61% to IGF2 (Ullrich *et al*, 1986). It consists of A and B chains homologous to the A and B chains of insulin but linked together by a C chain analogous to the C chain of preproinsulin and proinsulin and also containing a short D domain absent in insulin (Alvino *et al*, 2011). Seven of the twelve amino acids considered important for insulin receptor (IR) binding are conserved in both IGF1 and

IGF2 (Alvino *et al*, 2011). Like insulin, IGF1 has been shown to stimulate glucose uptake and storage as glycogen through the insulin/ IGF1 signalling (IIS) pathway (LeRoith, 2003). It has been proposed that a splice variant of the insulin receptor, IR-A has high affinity for IGF2, but that this interaction mediates predominately proliferative effects rather than the metabolic effects produced by insulin stimulation of IR-B (LeRoith, 2003).

1.6 *IGF1 receptor*

The IGF1 receptor (IGF1R) is structurally and functionally related to the insulin receptor. Both are transmembrane receptor tyrosine kinases consisting of two extracellular α subunits and two transmembrane β subunits linked together by disulphide bonds (LeRoith, 2003). IGF or insulin binding to the extracellular α subunits of the receptor induces a conformational change in the receptor enabling autophosphorylation of tyrosine residues in the intracellular β subunits (Schmelzle *et al*, 2006). Insulin binds with high affinity to the insulin receptor and with lower affinity to the IGF1R whereas IGF1 has the opposite affinities (Ullrich, 1986). Major effects of IGF1 include promotion of cell survival and inhibition of apoptosis (Vincent and Feldman, 2002). Stimulation of the IGF1R can promote cell proliferation and contact with the extracellular matrix (Vincent, 2002). Due to the high level of structural homology between these receptors, αβ hemireceptors can form in tissues which express both receptors. The physiological response induced by such hybrids is variable depending on the isoform of each subunit involved (LeRoith, 2003).

1.7 IIS pathway

Insulin and IGF1 signalling is transduced by intracellular second messengers within the insulin/IGF-1 signalling (IIS) pathway. These include the phosphatidylinositol 3 kinase (PI3K), mammalian target of rapamycin (mTOR) and mitogen activated protein kinase (MAPK) pathways.

1.8 PI3K pathway

Following activation of the IR, the PI3K signalling pathway, an intracellular phosphorylation cascade, illustrated in Fig 1.1 below, is initiated:

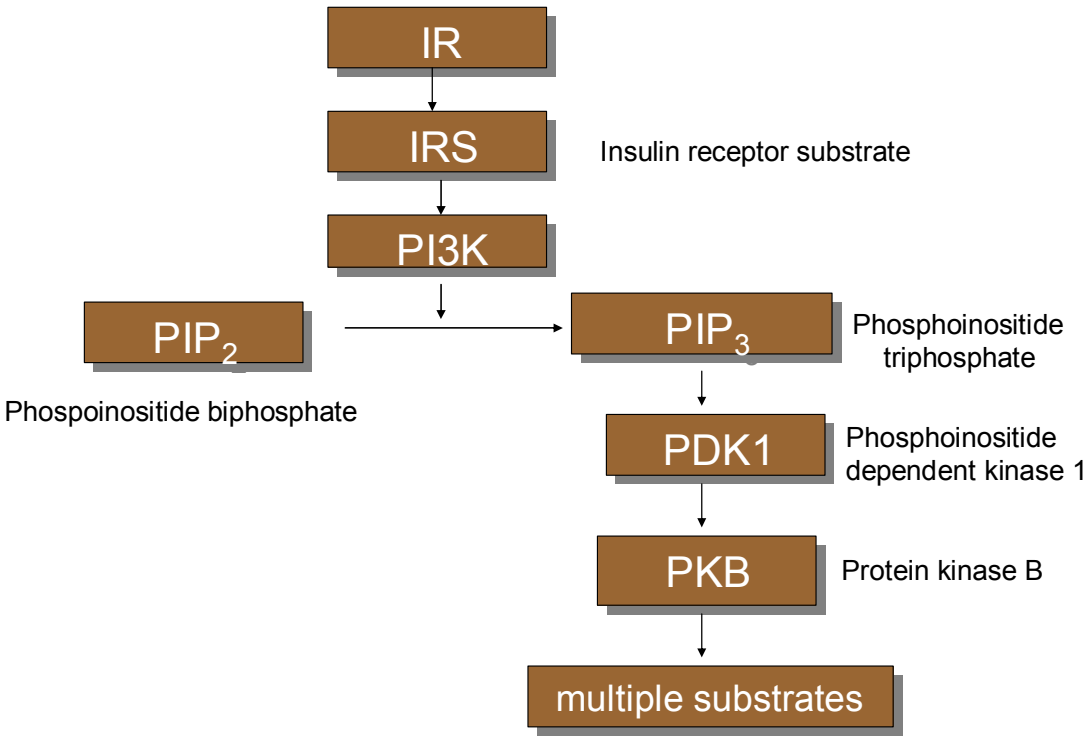


Fig 1.1: Schematic of the main insulin signalling cascade

Binding of insulin to the insulin receptor results in recruitment of IRS proteins and PI3K. PI3K catalyses phosphorylation of phospholipids. This results in recruitment then activation of PDK1 and PKB. PKB, in turn, phosphorylates a variety of substrates mediating the pleiotropic action of insulin.

1.8.1 Insulin receptor substrate

The activated IR phosphorylates specific tyrosine residues on insulin receptor substrates (IRS) that act as docking sites for additional signalling proteins (Lee and White, 2004; Taniguchi *et al*, 2006). The tyrosine phosphorylation sites interact with downstream substrates containing sarcoma (src) homology 2 (SH2) domains such as PI3K (Lee and White, 2004; Taniguchi *et al*, 2006). At least six insulin receptor substrate (IRS) proteins have been identified (Lee and White, 2004; Taniguchi *et al*, 2006). All IRS proteins have a pleckstrin homology (PH) domain and a phosphotyrosine binding (PTB) domain near the amino (NH_2) terminal which interacts with the IR (Lee and White, 2004; Taniguchi *et al*, 2006). IRS1 and IRS2 are widely expressed, whilst IRS3 is a rodent specific IRS and IRS4 is found in thymus, brain and kidney cells of humans and mice (Lee and White, 2004; Taniguchi *et al*, 2006). The functions and expression of IRS5 and IRS6 are less well understood. The carboxy tail is of variable length, longest in IRS1 and IRS2, and contains tyrosine and serine phosphorylation sites (Lee and White, 2004). IRS1 deficient mice have been shown to display growth retardation and mild insulin resistance whereas IRS2 deficient mice display a more severe diabetic phenotype due to impaired β cell function (Withers *et al*, 1998).

1.8.2 Phosphatidylinositol 3 kinase

Phosphatidylinositol 3 kinase (PI3K) phosphorylates the 3’ hydroxyl (OH) group of the inositol ring of the inositol phospholipids, phosphatidylinositol (PI), phosphoinositide monophosphate (PIP) and phosphoinositide

biphosphate (PIP_2) to produce PIP, PIP_2 and phosphoinositide triphosphate (PIP_3) respectively (Cantrell, 2001; Taniguchi *et al*, 2006). There are three classes of PI3K (Cantrell, 2001). Class I PI3Ks consist of a regulatory and a catalytic subunit, each of which occurs in several isoforms resulting in different combinations of PI3K due to various dimerizations of several regulatory and catalytic subunits (Cantrell, 2001; Taniguchi *et al*, 2006). These PI3Ks preferentially phosphorylate PIP_2. Class II PI3Ks have a catalytic but no regulatory domain and preferentially phosphorylate PI and PIP (Cantrell, 2001). Class III PI3Ks, like class I, are heterodimeric but are involved in protein trafficking via vesicular transport (Abe *et al*, 2009) and only phosphorylate PI. Only class I PI3Ks respond to extracellular stimuli (Cantrell, 2001) such as insulin signalling (Taniguchi *et al*, 2006).

Some selective or specific protein kinase inhibitors are available that inhibit PI3K. 2- (- 4- morpholinyl)- 8- phenyl- 4H- 1- benzoptran- 4- one (LY294002) has been proposed as PI3K selective (Davies *et al*, 2000) and LY294002 and PI-103 to be selective for class I (Vlahos *et al*, 1994). 3- methyl adenine has been suggested to selectively inhibit class III (Egami and Araki, 2008). Wortmannin has been proposed to be PI3K specific (Davies *et al*, 2000) with selectivity for class I and II (Egami and Araki, 2008).

1.8.3 Phosphatidylinositols and phosphoinositides

Phosphatidylinositols (PIs) are components of the cytosolic surface of the cell membrane. They consist of a glycerol backbone, two fatty acid side chains, a phosphate group and an inositol group. The inositol group can be phosphorylated by PI3Ks on the 3', 4' and 5' OH groups (Paolo and Camilli,

2006). The 1' carbon is usually bound to the phosphate group and the 2' and 6' OH groups are generally not phosphorylated due to steric hindrance (Mathews *et al*, 2005). Phosphorylated forms of PI are referred to as phosphoinositides. Activation of the IR leads to the recruitment of PI3K to the cell membrane where it can phosphorylate PIs, ultimately generating PIP_3 (Taniguchi, 2006). Proteins with PH domains such as phosphoinositide dependent kinase (PDK1) can bind to PIP_3 and become localised to the cell membrane in the process (Taniguchi *et al*, 2006).

1.8.4 Phosphoinositide dependent protein kinase 1

Phosphoinositide dependent protein kinase 1 (PDK1) consists of a catalytic kinase domain and a PH domain (Alessi *et al*, 1997). The PH domain interacts with phosphoinositides (Biondi *et al*, 2000) and the kinase domain with various substrates including protein kinase B (PKB), phosphorylating and therefore activating it (Alessi *et al*, 1997). A hydrophobic pocket within the kinase domain of PDK1 interacts with a PDK1 interacting fragment (PIF) of PKB, acting as a docking site (Biondi *et al*, 2000) facilitating phosphorylation of PKB at Thr308 before being released into the cytoplasm in its active form (Alessi *et al*, 1997).

1.8.5 Protein kinase B

There are three isoforms of protein kinase B (PKB) – PKBα, β and γ. PKB consists of an N-terminal PH domain, a catalytic domain and a short C-terminal tail (Alessi *et al*, 1996). In addition to interacting with a PDK1 'docking site', the PH domain of PKBα can also interact with PIP_3 and be

recruited to the cell membrane and undergo activation by PDK1 mediated phosphorylation of Thr 308 and Ser 473 (Shaw *et al*, 1998).

There are various inhibitors for different isoforms of PKB. The commercially-available Akti, is a PKB inhibitor that has been found to abolish PKB activity in L6 myotubes which predominately express PKBα and γ (Green *et al*, 2008). Akti 1/2 has been shown *in vitro* to only inhibit PKB α and β (Logie *et al*, 2007). Akti-1 inhibits only PKBα (Barnett *et al*, 2005) and Akti-2 only PKBβ (DeFeo-Jones *et al*, 2005). Isothiocyanates, a group of compounds found in various vegetables have been suggested to be inhibitors of PKBγ (Sharma *et al*, 2009). PKB inhibitors can be used to determine whether this kinase mediates downstream cellular responses rather than other signalling pathways such as those regulated by mitogen activated protein kinase (MAPK) or mammalian target of rapamycin (mTOR).

1.8.6 Mammalian target of rapamycin (mTOR)

mTOR was originally identified as a target of the immunosuppressant drug rapamycin. It is regulated by various growth factors including insulin in a normal physiological setting (Chung *et al*, 1994). Activation of mTOR ultimately regulates protein translation, for example by inducing S6 protein phosphorylation (LaPlante and Sabatini, 2009).

1.9 Forkhead box subtype O

Forkhead box proteins are a group of proteins characterised by a conserved DNA binding domain, the Forkhead box, or Fox (Clark *et al*, 1993). The first of these proteins was identified in the fruit fly *Drosophila melanogaster* (Liu and Lehman, 2008) and named after the ectopic head structures which

developed as a consequence of mutation of the gene which encodes the protein (Jurgens and Weigel, 1988). This and subsequent Fox proteins which have been identified have been assigned to a subclass - A to O - and proteins within each subclass have been allocated a number i.e. FoxO1. Abbreviations for Fox contain all upper case letters for humans i.e. FOXO1 but only the first letter is upper case for murine Foxo1 (Kaestner *et al*, 2000). FOXO proteins are transcription factors which are phosphorylated and as a consequence excluded from the nucleus and therefore inactivated, 'downstream' of the insulin receptor or IGF-1 receptor. Here 'downstream' simply means that FOXO regulation occurs at the foot of the intracellular pathway triggered by insulin binding to its receptor. A FOXO homologue has been identified in *Drosophila* (dFOXO) and in *C.elegans* (dauer arrest phenotype 16 - DAF16) (Van Der Heide *et al*, 2004). The relevant pathways are highly conserved throughout animal evolution, as shown in Fig 1.2:

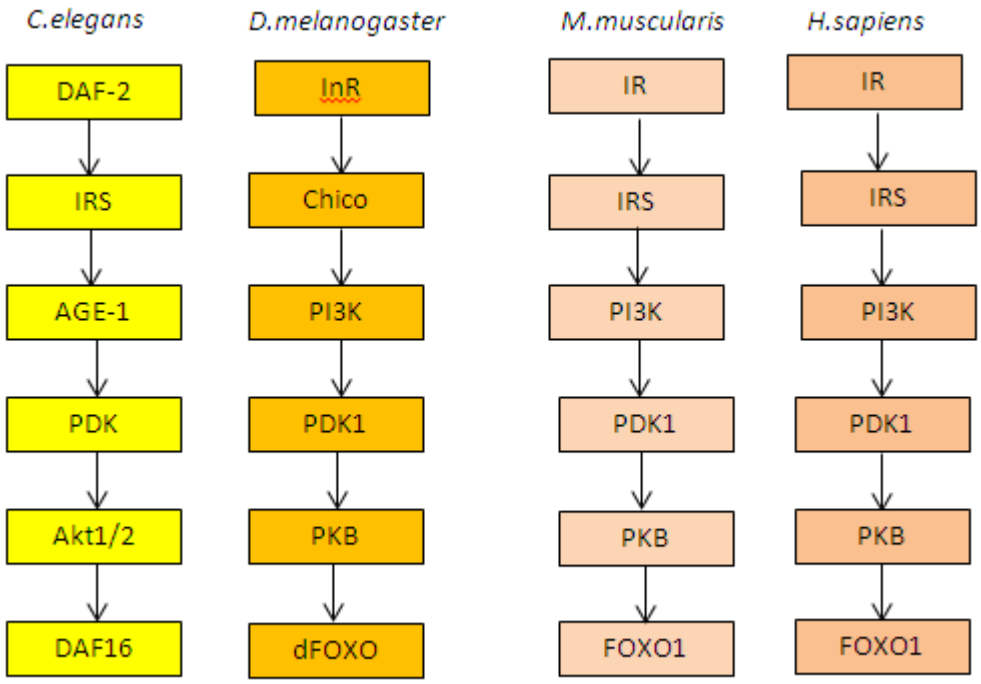


Fig 1.2: Analogous insulin signalling pathways in the nematode, fruit fly, mouse and human (Adapted from Woods *et al*, 2002). There is evolutionary conservation of proteins at each step in the pathway.

1.9.1 Regulation of FOXO1

FOXO1 has been shown to be regulated by phosphorylation (Biggs *et al*, 1999) and nuclear/ cytosolic shuttling (van der Heide e*t al*, 2004).

1.9.1.1 Phosphorylation of FOXO1

FOXO1 has been shown to be phosphorylated and inactivated by PKB in response to insulin or IGF1 at specific residues (see figure 1.3) which lie within Arg–Xaa–Arg–Xaa–Xaa–Ser/Thr motifs (Rena *et al*, 1999). This phosphorylation results in nuclear exclusion and therefore inactivation of the transcription factor (Rena *et al*, 2001).

It has been proposed that PKB mediated phosphorylation of Ser256 induces a conformational change in the protein which enables Thr24 and Ser319 phosphorylation (Rena *et al*, 2001). Phosphorylation of Ser319 primes the molecule for Ser 322 followed by Ser 325 phosphorylation most likely catalysed by casein kinase 1 (CK1) (Rena, *et al* 2002). This hierarchal sequence of phosphorylation events is illustrated in Fig 1.3:

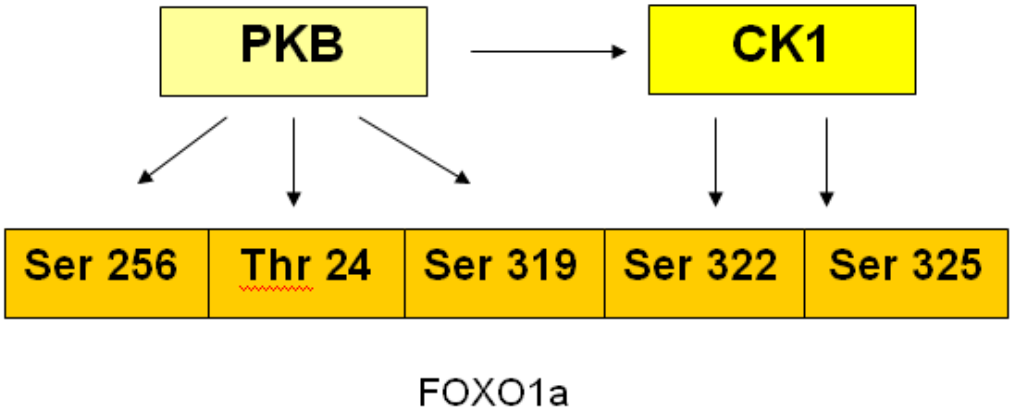


Fig 1.3: **Schematic of FOXO1a phosphorylation**
From left to right, the sequence of phosphorylation events which result in exclusion of FOXO1 from the nucleus and therefore result in inactivation of the transcription factor.

1.9.1.2 FOXO1 inactivation by nuclear exclusion

An intracellular 'shuttling' system exists which confines FOXO1 either to the cytoplasm, effectively inactivating it, or to the nucleus, which allows FOXO1 to bind to specific DNA sequences and enables transcription of genes believed to be involved in glucose regulation such as PEPCK. Only proteins with a molecular mass of less than 50 kilodaltons (kDa) or a diameter of less than 9 nanometres (nm) diameter can passively diffuse through the nuclear pore. Larger molecules require active transport mechanisms. Several nuclear hormone receptors have been found to bind to FOXO1a, influencing its

nuclear exclusion and entry. These include estrogen, progesterone and androgen receptors as well as glucocorticoid and thyroid hormone receptors (Van der Heide *et al*, 2004).

FOXO1 localisation in response to insulin is regulated by phosphorylation (Rena *et al*, 2001). Phosphorylation of Thr24 has been proposed to enhance nuclear exclusion of FOXO1 by binding to 14-3-3 proteins, probably by reducing the affinity for DNA binding (Van der Heide *et al*, 2004). A cluster of phosphorylated residues including Ser319, Ser322 and Ser325 is also critical for nuclear exclusion, as FOXO6, which lacks these residues but retains other regulatory features of FOXO1, is restricted to the nucleus (Jacob *et al*, 2003). Mutation of these serines in FOXO1 retards but does not prevent nuclear exclusion (Rena, *et al* 2002) however, suggesting that the unphosphorylated motif supports a reduced rate of nuclear exclusion. Thus, phosphorylation of this cluster may reduce access to the nuclear localisation signal (NLS) or increase access to the nuclear exclusion sequence (NES) of the protein (Woods *et al*, 2002), or alter interaction with the nuclear hormone receptors described above.

1.9.2 Functions of FOXO1

Various studies have helped confirm the physiological roles of FOXO1

1.9.2.1 Development

By embryonic day 9.5 (E9.5), *Foxo1*$^{-/-}$ mouse embryos have been found to be approximately half the size of their *Foxo1*$^{+/+}$ and heterozygous littermates and to have cardiac, aorta and carotid abnormalities as well as poor vascular development of the yolk sac (Hosaka *et al*, 2004). All these mice died between E9.5 and E10.5 (Hosaka *et al*, 2004), suggesting a role for Foxo1 in vascular development.

1.9.2.2 Aging

It has been shown that the IIS pathway influences lifespan. *C. elegans* can respond to environmental stress such as food limitation by arresting maturation during an early alternative post-embryonic larval stage called dauer therefore these are longer lived than non dauer nematodes (Ogg *et al*, 1997, Lin *et al,* 1997). *C. elegans* with impaired IIS function such as those carrying *daf-2* mutations have been shown to be longer lived than wild type (WT) phenotypes (Ogg *et al*, 1997, Lin *et al* 1997). Equivalent studies on mammalian FOXOs are made difficult by the existence of multiple FOXO isoforms; however evidence that female *Irs1*$^{-/-}$ mice are long lived and *Irs2*$^{-/-}$ mice are short lived is consistent with a role for mammalian FOXOs in organismal longevity (Selman *et al*, 2008). It is important to remember however that reduced IIS in mammals ultimately risks diabetes and shorter longevity, which is the phenotype of *Irs2*$^{-/-}$ mice.

1.9.2.3 Metabolic functions of FOXO1a

FoxO1 has been shown to be involved in both the development of adipose tissue and in the upregulation of gluconeogenesis (covered in more detail in the following section) (Gross *et al*, 2008). In the central nervous system (CNS), it has also been shown to mediate central actions of both insulin and leptin (an endogenous appetite suppressant) within the hypothalamus, influencing feeding behaviour. FoxO1 is also expressed in pancreatic β cells where it is thought to influence β cell proliferation (Glauser and Schlegel, 2007).

1.9.2.3.1 Regulation of gluconeogenesis by FOXO1a

Gluconeogenesis is the production of new glucose from noncarbohydrate substrates such as amino acids, lactate and pyruvate (Devlin, 2011). Phosphoenolpyruvate carboxylase (PEPCK) has been shown to be a rate limiting enzyme in this pathway and as such has undergone extensive study. For example Scott *et al*, (1998) found repression of PEPCK to be glucose dependent but insulin independent, suggesting a negative feedback mechanism for glucose regulation not dependent on the insulin response. Another key enzyme, glucose 6-phosphatase (G6Pase) is involved in the final step of gluconeogenesis and catalyses the conversion of glucose-6-phosphate to glucose (Sutherland *et al*, 2006). In a variety of experiments including genetic analyses, it has been suggested that increased expression of FOXO1 increases hepatic gluconeogenesis (Frescas *et al*, 2005; Nakae *et al*, 2002; Zhang *et al*, 2006). Nakae *et al*, for example, introduced a gene trap cassette into murine *Foxo1* and demonstrated that the diabetic

phenotype resulting from *Insr* haploinsufficiency is partly rescued in *Insr* $^{+/-}$ *Foxo1*$^{+/-}$ heterozygotes through suppression of gluconeogenic gene expression (Nakae *et al*, 2002). Consistent with this, transgenic (TG) mice that express constitutively active hepatic FoxO1 have been noted to have elevated fasting glucose levels and impaired glucose tolerance in comparison to their WT counterparts (Zhang *et al*, 2006). It is important to note however that other factors besides FOXO1a also contribute to regulation of these genes (Barthel *et al*, 2001; Lochhead *et al*, 2001; Logie *et al*, 2007). Gluconeogenesis is illustrated schematically in Fig 1.4:

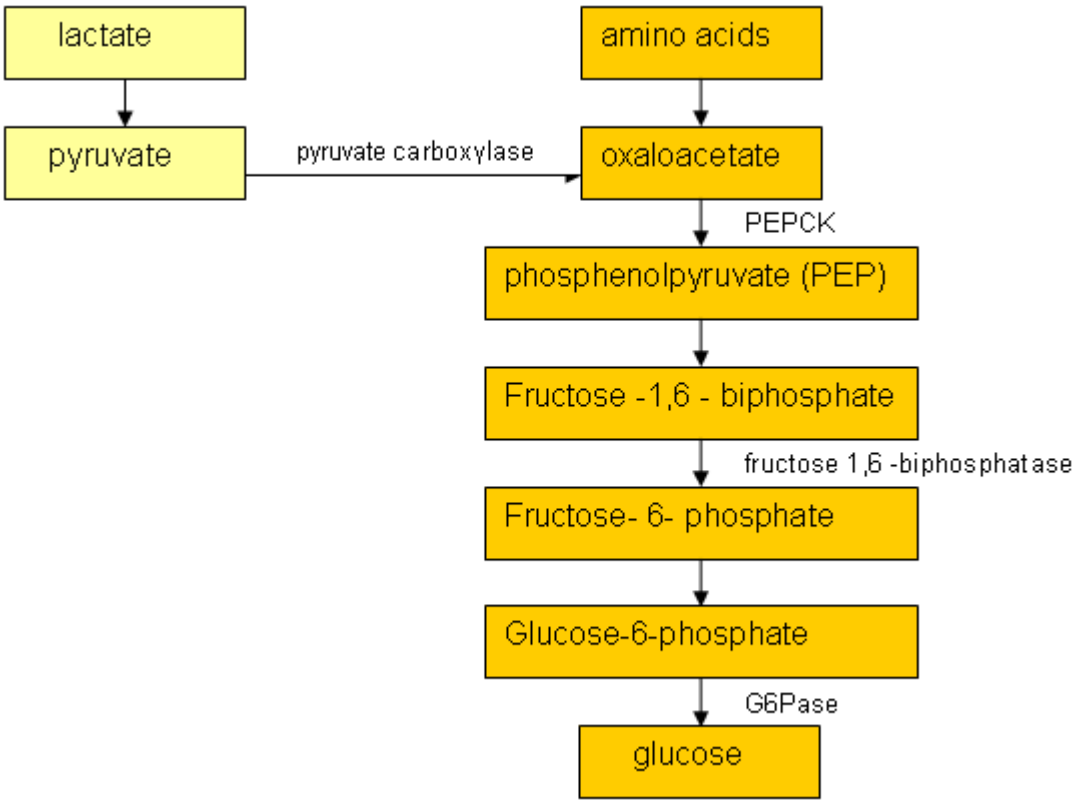


Fig 1.4: Hepatic gluconeogenesis

As described by Devlin, 2011

1.10 Regulation of ribosomal protein S6

Phosphorylation and therefore activation of S6 by S6K is believed to be important in regulation of mRNA translation and therefore cell growth and proliferation (Laplante and Sabatini, 2009). Treatment of cells with insulin induces S6 phosphorylation which is reduced in the presence of wortmannin, suggesting involvement of PI3K within the insulin signalling pathway (Pullen *et al*, 1998). Consistent with this, genetic modification of PDK1 demonstrated that this kinase is also active upstream of S6 phosphorylation (Collins *et al*, 2003). The mechanism of PDK1/PKB dependent activation of S6K is complex, has only been resolved more recently (Laplante and Sabatini, 2009) and is illustrated schematically in Fig 1.5:

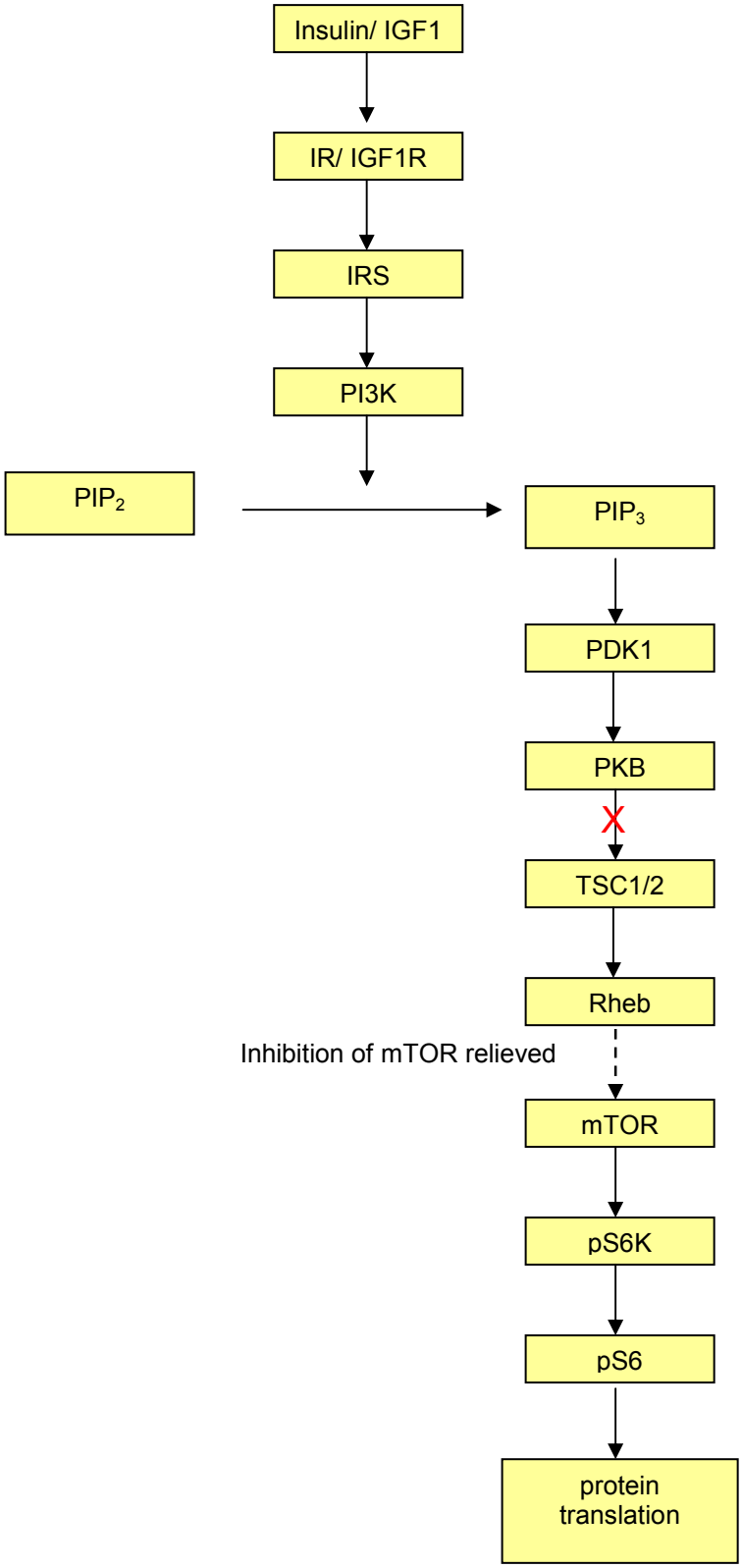


Fig 1.5: Schematic representation of regulation of S6

As described by Huang and Manning, 2008, resulting increased protein translation. The red cross denotes inhibition by PKB. Some steps are omitted for clarity.

1.10.1 Tuberous sclerosis complex (TSC).

TSC 1 and *TSC2* are tumour suppressor genes mutated in tuberous sclerosis complex syndrome (Huang and Manning, 2008), causing benign tumours to grow in the brain and other major organs such as the skin, kidneys, heart and lungs. This results in a combination of symtpoms including seizures, developmental delay, skin abnormalities and kidney disease (National Institutes of Health). TSC1/2 regulate mTOR indirectly through another protein, Rheb (Huang and Manning, 2008).

1.11 Cell signalling responses to insulin sensitising agents

Insulin sensitising agents such as metformin and thiazolidinediones do not act directly to induce IIS like insulin does. Pathways regulated by these agents are discussed below.

1.11.1 Adenosine monophosphate activated protein kinase

Adenosine monophosphate activated protein kinase (AMP activated protein kinase) has been described as a heterotrimeric enzyme consisting of a catalytic (α) and two regulatory subunits (β and γ) (Zhang *et al*, 2009). Isoforms containing the α_2 subunit are more AMP sensitive than those containing the α_1 (Winder and Hardie, 1999). AMPK is activated by cellular stresses that deplete ATP (Winder and Hardie,1999). It acts as an energy sensor within the cell and as a result of adenosine triphosphate (ATP) depletion, upregulates ATP producing pathways and downregulates ATP consuming pathways (Hawley *et al*, 2002) such as those regulated by acetyl CoA carboxylase (Abu-Elhelga *et al*, 2003). Conditions that increase intracellular AMP/ADP ratio therefore lead to AMPK activation (Zhang *et al*,

2008). The γ subunit of AMPK contains two pairs of cystathione β synthase (CBS) domains, each pair making up a Bateman domain. Binding of one AMP to one Bateman domain cooperatively increases binding affinity of the second (Adams *et al*, 2004). This causes a conformational change in the γ subunit which exposes the catalytic domain of the α subunit enabling phosphorylation by an upstream AMPKK (Hawley *et al*, 1996). A simplified version of this pathway is illustrated in Fig 1.6:

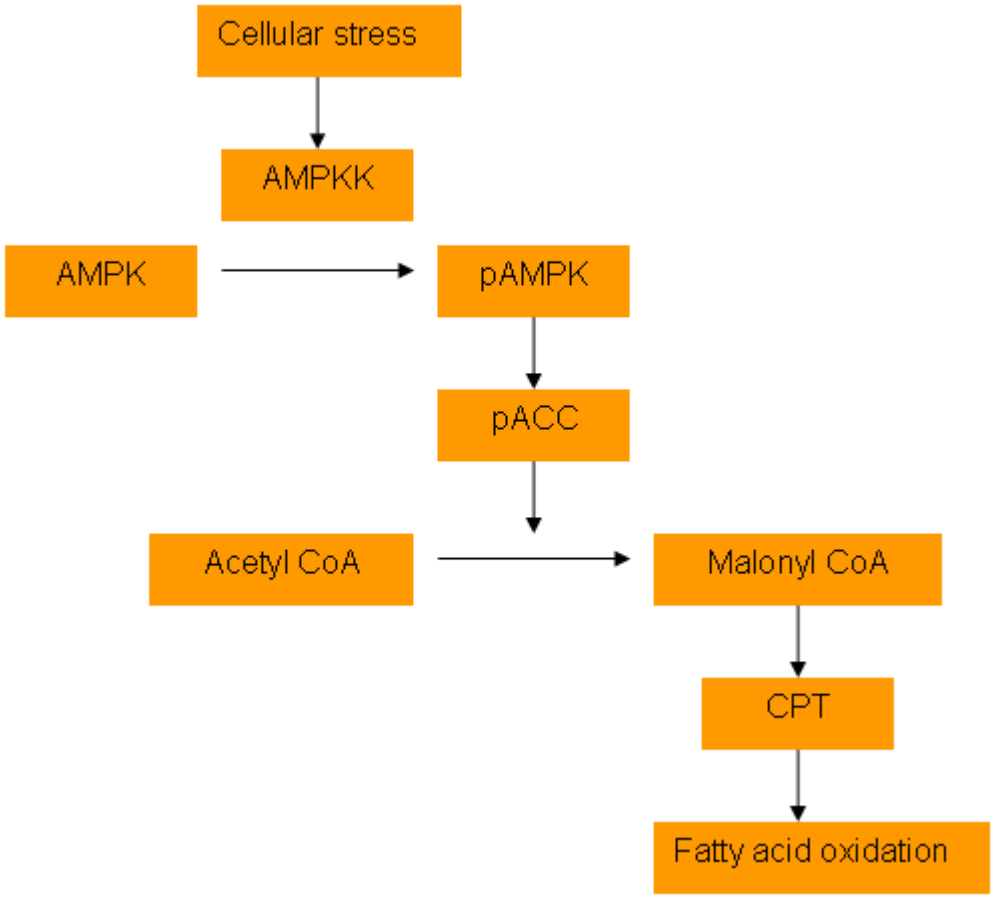


Fig 1.6: Schematic of AMPK signalling

Activation of AMP activated protein kinase (AMPK) results in increased production of ATP through fatty acid oxidation (Abu-Elhelga *et al*, 2003).

Various endogenous AMPK activators have been proposed. In addition to ACC, 3- OH- 3 methylglutaryl CoA reductase (HMGR), an enzyme involved in sterol production has also been proposed as a potential target of AMPK. In

adipose tissue, hormone sensitive lipase (HSL), involved in fatty acid synthesis, is an indirect substrate for AMPK as a consequence of phosphorylation of ACC (Winder and Hardie, 1999). AMPK has also been suggested to downregulate PEPCK and G6Pase (Lochhead *et al*, 2000). However, a later study by Foretz, *et al* 2010 suggested only AMP was necessary to inhibit hepatic glucose production, as ZMP inhibited cAMP mediated expression of G6Pase and PEPCK (Foretz *et al*, 2010).

Exogenous AMPK activators include metformin, used clinically as an antihyperglycemic agent (Zhou, 2001), thiazolidinediones (Le Brasseur *et al*, 2006) as do many natural products, including green tea catechins (Collins *et al*, 2007), and berberine, an alkaloid extracted from *Rhizoma coptidis,* a herb used in traditional Chinese medicine which inhibits mitochondrial activity and therefore increases AMP/ADP ratio (Yin *et al*, 2008).

AICAR monophosphate (ZMP), is a commercially available AMP analogue, therefore it can also bind to the Bateman domain, resulting in activation of AMPK. As an AMP analogue, this drug is likely to regulate other AMP-sensitive proteins besides AMPK. Cannabinoids and ghrelin both have central and peripheral metabolic and cardiac effects via AMP-activated protein kinase (Kola *et al*, 2005)

1.11.2 Acetyl CoA carboxylase

Acetyl CoA carboxylase (ACC) carboxylates acetyl CoA to form malonyl CoA, a key molecule involved in the control of fatty acid synthesis and oxidation. Acetyl CoA is probably the best characterised substrate and downstream readout for AMPK activation (Hawley *et al*, 2010). ACC is inactivated by phosphorylation and activated by dephosphorylation on serine

residues 23, 25, 29, 76, 77, 95 and 1200 (Mabrouk *et al*, 1990). Glucagon, produced in response to hypoglycaemia, has been shown to induce phosphorylation of ACC, whereas insulin has the opposite effect. Two isoforms of ACC have been identified, ACC1 localised to the cytosol and ACC2 on the mitochondrial membrane. Malonyl CoA synthesised by ACC1 is thought to be involved in fatty acid synthesis and inhibition of this isoform results in decreased consumption of ATP for fatty acid synthesis. Malonyl CoA synthesised by ACC2 is thought to be involved in fatty acid oxidation and inhibition of this enzyme results in increased production of ATP through fatty acid oxidation (Abu-Elhelga *et al*, 2003).

1.12 Factors leading to Diabetes

Diabetes is a term use to describe a group of conditions characterised by hyperglycemia or elevated blood glucose levels. Type 1 and type 2 are the most commonly observed types of diabetes. Type 1 diabetes mellitus (T1DM) is a consequence of autoimmune destruction of pancreatic β cells so the patient does not produce insulin. In type 2 diabetes mellitus (T2DM) the patient produces insulin but liver, muscle and fat cells are unresponsive to it, resulting in hyperglycaemia.

1.12.1 Insulin resistance

Various hypotheses have been proposed as to why obesity and T2DM should frequently exist comorbidly but the molecular basis of insulin insensitivity is most likely multifactorial.

1.12.2 Free fatty acids

Obesity and increased plasma free fatty acid (FFA) concentrations are associated with insulin resistance (Shulman, 2000). Randle and co-workers first showed that fatty acids competed with glucose for substrate oxidation, speculating that increased fat oxidation causes insulin resistance (Randle *et al*, 1963). Because of the close proximity of abdominal fat to the hepatic portal vein, fatty acids within these cells can drain directly into the portal venous system and thereafter into the general circulation. This, according to the lipid supply hypothesis of insulin resistance, could result in the impaired insulin mediated glucose uptake in insulin sensitive tissue that contributes to conditions such as metabolic syndrome and T2D (Greenfield and Campbell, 2004). In a study by Roden *et al*, glucose and insulin levels of healthy human subjects were maintained under a hyperinsulinemic euglycemic clamp. A lipid infusion administered increase in circulating fatty acid concentration caused a reduction in insulin stimulated muscle glycogen synthesis (Roden *et al*, 1996). Attributed by Randle to direct inhibitory effects of FFAs on metabolic enzymes, these more recent studies emphasised the importance of FFAs on cell signalling mechanisms (Shulman, 2000).

1.12.3 Inflammation

Increased levels of chronic inflammation are another possible factor underlying insulin resistance. Much of this literature is focussed on the immunological cytokine- tumour necrosis factor α (TNFα), which is involved in systemic inflammation. Increased TNF α results in activation of inhibitory κ kinase (IKK), which consists of heterodimers of IKKα and IKKβ. Nuclear factor κ light chain enhancer of activated B cells (NF–κB) is a transcription factor excluded from the nucleus by inhibitory κβ (IkB). IKK phosphorylates and therefore degrades and inactivates IκB resulting in translocation of NF-κB to the nucleus where it can initiate the synthesis of molecules involved in the immune response (Yinn *et al*, 1998). This sequence of events is illustrated diagramatically in fig 1.7:

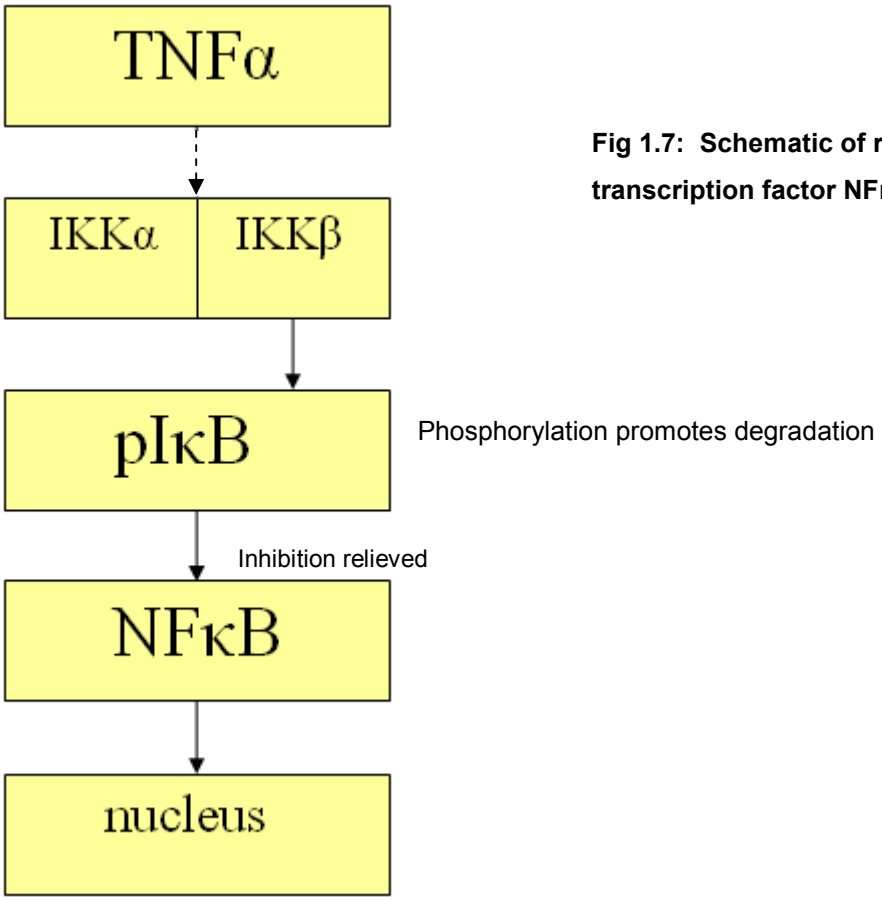


Fig 1.7: Schematic of regulation of transcription factor NFκB

High doses of sodium salicylate and related drugs have been known for many years to reduce glucosuria in some patients with diabetes (Williamson, 1901). Much more recent data suggested that these antihyperglycaemic effects depend on the antiinflammatory effects of salicylate and acetylsalicyclic acid, mediated at least in part by inhibition of the kinase IKKβ (Yin *et al*,1998). The effects of salicylate pretreatment on fat induced insulin resistance were measured in hyperinsulinemic euglycemic clamped rats. Salicylate pretreatment prevented the lipid infusion induced reduction of PI3K activity observed in untreated animals. Also, $IKKB^{(-/-)}$ mice did not exhibit decreased insulin stimulated glucose uptake in contrast to wild type, suggesting interaction between these pathways (Kim *et al*, 2001). Other pro-inflammatory signalling pathways have been implicated in insulin resistance

and T2DM. For example, c-Jun N terminal kinases (JNKs) phosphorylate the transcription factor c-Jun in response to a variety of stimuli including inflammatory signals (Kynakis and Avrush, 2001). JNK is activated by cytokines, involved in defence and also by free fatty acids (Hotamsligil, 2006). Activation of JNKs in insulin target cells results in phosphorylation and therefore inactivation of IRSs and they are also required for the accumulation of visceral fat (Solinas *et al*, 2007).

In summary, activation of JNK or IKK can phosphorylate IRS, perhaps suppressing insulin responses directly. However, excess free fatty acids (FFA) can also induce an inflammatory type response that may contribute to insulin resistance. For example cytokines have been shown to be overexpressed in adipose tissue in obese mice and humans (Wellen and Hotsamligil, 2005). It is therefore possible that this type of response to excessive levels of FFAs and other nutrients can lead to inflammation which in turn leads to phosphorylation of IRS (Hotamsligil, 2006). A simplified version of these events is illustrated in Fig 1.8:

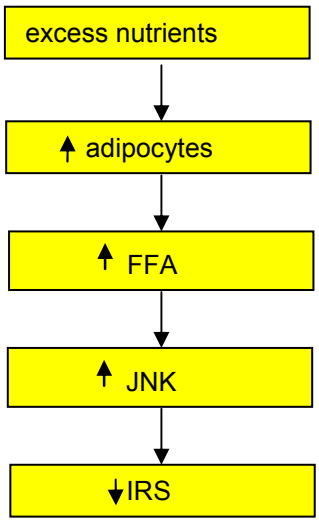


Fig 1.8: A possible model of inflammation contributing to insulin resistance.
Adapted from (Hotamsligil, 2006)

1.12.4 Genetic predisposition

In the 1960s, the theory that a naturally selected 'thrifty' genotype which enabled efficient utilisation and storage of food and later conferred vulnerability to insulin insensitivity with the advent of a plentiful supply of food was proposed (Neel,1962). Later the 'thrifty' phenotype hypothesis emerged which suggested *ante natal* nutritional status to indicate the nutritional environment into which the fetus would be born and therefore influence later vulnerability to insulin sensitivity (Hales, 1997; Hales and Barker, 2001).

1.13 Type 2 diabetes mellitus

This is a disorder of glucose metabolism characterised by insulin resistance of muscle, fat and liver cells. Before the onset of diabetes, insulin resistance may be overcome by increased insulin production and release by pancreatic β cells but this compensatory mechanism eventually breaks down and there is destruction of these cells. Type 2 diabetes mellitus (T2DM) has been described as an impending global epidemic (Mulder, 2010). The World Health Organisation (WHO) estimate that by the year 2030, 350 million

people will suffer from this condition worldwide and are seeking to improve current risk prediction models (Collins *et al*, 2011).

1.14 Conditions associated with T2DM

Various conditions have been associated with T2DM, including obesity (Li *et al*, 2011), cancer (Stocks *et al*, 2009), cardiovascular disease (Lebovitz, 2006), and Alzheimer's Disease (Han and Li, 2010).

1.14.1 Cancer

In 2009, a prospective study by Stocks *et al* showed a link between hyperglycemia and increased risk of cancer. It was proposed by the group that elevated blood glucose levels may contribute to this both directly and indirectly. Directly by providing an easily accessible energy substrate for tumour cells and indirectly by increasing IGF1 levels (Stocks *et al*, 2009). Metformin use has been associated with a reduced risk of cancer, possibly due to activation of AMPK (Libby *et al*, 2009). Activated AMPK activates the tumour suppressor gene p53 which can inhibit mTOR via activation of TSC1 and TSC2 and therefore inhibit cell growth (Vousden and Ryan, 2009).

1.14.2 Cardiovascular and vascular disease

Both micro and macrovascular pathologies are associated with T2DM, including retinopathy (Creager and Luscher, 2003) and ischaemic heart disease (Despres *et al*, 1996), respectively. Many theories have been proposed to explain this, including that insulin may upregulate nitric oxide synthase (NOS) production in endothelial cells and that insulin resistance could therefore lead to reduced nitric oxide (NO) mediated vasodilation in

these cells (Campia *et al*, 2004). This could at least partly explain the hypertension and atherosclerosis which often appears to contribute to cardiovascular conditions in T2DM (Campia *et al*, 2004). Hyperglycemia has also been shown to raise diacylglycerol (DAG) levels. This activates PKC which subsequently inhibits PKB which can therefore no longer phosphorylate NOS (Creager and Luscher, 2003).

1.14.3 Alzheimer's Disease

Epidemiological studies have suggested a link between T2DM and Alzheimer's Disease (AD) (Cole *et al*, 2007). This is a neurodegenerative disorder characterised by global cognitive decline. The two most frequently documented pathological hallmarks of this condition are Aβ plaques resulting from the abnormal cleavage of the amyloid precursor protein (APP) and neurofibrillary tangles resulting from phosphorylation of the microtubular protein, tau. Potential molecular connections between T2DM and AD include glycogen synthase kinase 3 (GSK3), which is a kinase that lies downstream of the IR (Taniguchi *et al*, 2006). Following activation of the IR, GSK3 is phosphorylated and therefore inactivated by PKB, facilitating glycogen production and storage (Taniguchi *et al*, 2006). If not inactivated in this way it has also been shown, *in vitro,* to increase Aβ production and to phosphorylate tau, resulting in these characteristic plaques and tangles (Cole *et al*, 2007).

1.15 Diagnosis and treatment of T2DM

Accurate diagnosis and classification of diabetes is important with respect to clinical decisions made and lifestyle advice offered.

1.15.1 Diagnosis

Diagnostic tools available include measurement of blood glucose levels and consideration of medical and family history (SIGN guideline 116). Diagnosis is usually confirmed by hyperglycemia followed by a glucose tolerance test (SIGN guideline 116; Craig 2009). Age and severity of onset is usually later and milder than in T1DM and rate of onset of the condition and progression of symptoms is usually slower (Craig *et al*, 2009). However, classifying the type of diabetes could ideally be facilitated by measuring autoantibody markers (Craig *et al*, 2009). Measurement of C-peptide is a potential useful indicator of endogenously produced insulin levels for diagnosis of T2DM (Craig *et al*, 2009). Glycosylated haemoglobin (HbA1c) is used to monitor progression of diabetes, as a measure of average blood glucose level over the past 2-3 months (SIGN guideline 116). T2DM is often accompanied by obesity and acanthosis nigricans, a hyperpigmentation observed within skin folds. A family history of T2DM is often also present (Craig *et al*, 2009).

1.15.2 Treatment

Various pharmacological interventions are currently available for the treatment of T2DM (SIGN guideline 116). These consist mainly of oral antihyperglycemic agents and insulin.

1.15.2.1 Sulphonylureas

Insulin secretagogues, such as sulphonylureas (SURs), increase insulin secretion from the pancreatic β cells but pose a risk of hypoglycaemia. Drugs in this class include glyburide and glipizide (Nyenwe *et al*, 2011).

1.15.2.2 Thiazolidinediones

Thiazolidinediones (TZDs) bind to peroxisome proliferator activated receptor (PPAR) transcription factors in the nucleus and therefore activate the transcription of various proteins (Smith, 2002). These include increased expression of glucose transporters such as GLUT1 and 4, upregulation of IRS, decreased activity of PEPCK and increased levels of lipoprotein lipase, upregulating plasma triglyceride clearance (Smith, 2002). This results in increased insulin sensitivity and glucose utilisation and downregulation of gluconeogenesis (Smith, 2002). Endogenous activators of PPARs include some molecules involved in insulin signalling and glucose and energy homeostasis (Smith, 2002). These include fatty acids (Schoojans *et al*, 1996) and acyl CoA derivatives (Bronfman *et al*, 1986). However, some of these TZDs have been implicated in fluid retention, congestive cardiac failure and osteoporosis (Nyenwe *et al*, 2011). As a result, there is only one TZD, pioglitazone, still in clinical use in the UK. Other studies indicate that there are important PPAR gamma independent targets of TZDs, including AMPK, which is also a target of metformin (Fryer *et al*, 2002).

1.15.2.3 α-glucosidase inhibitors

This group of compounds inhibit the action of α-glucosidase in the small intestine and reduce the absorption of glucose due to the reduction in breakdown of starch to glucose as α glucosidase in the small intestine would normally be involved in the catabolism of polysaccharides to monosaccharides (Nyenwe *et al*, 2011). This can, however, lead to excess glucose in the colon, which can cause problematic and often poorly tolerated gastrointestinal disturbances (Nyenwe *et al*, 2011). Hypoglycaemia as a consequence of poor glucose absorption can sometimes also be an issue and the dose and timing of drug application often requires to be considered on an individual basis (Nyenwe *et al*, 2011).

1.15.2.4 Biguanides

Biguanides such as metformin appear to act mainly by restoring suppression of hepatic gluconeogenesis which becomes abnormally elevated in T2D; however their mechanism of action remains a subject of ongoing investigation. Elevated muscle glucose uptake and decreased hepatic gluconeogenesis have been shown to contribute to the antihyperglycemic effects of metformin which have been proposed to be mediated via AMPK (Fryer *et al*, 2002) or possibly suppression of mTOR signalling (Kalendar, 2010). Recent studies indicate that AMPK is not required for effects of metformin on S6 (Kalendar *et al*, 2010; Kickstein *et al*, 2010), suggesting that these two pathways can operate independently. Metformin has also been proposed to inhibit gluconeogenesis by inhibition of complex 1 of the mitochondrial respiratory chain (Owen *et al*, 2000). This increases the AMP/

ATP ratio, which in turn activates AMPK (Foretz *et al*, 2010) Gastrointestinal side effects can be problematic (Nyenwe *et al*, 2011).

1.15.2.5 GLP1 agonists

Incretins increase post prandial insulin release from the pancreas. One such molecule is glucagon like peptide 1 (GLP1) (Drucker and Nauck, 2006). Exenatide and liraglutide are clinically available GLP1 agonists administerable subcutaneously (http://www.nice.org.uk/guidance/index).

1.15.2.6 SGLT2 inhibitors

Reabsorption of glucose occurs in the proximal tubule of the kidney by sodium coupled glucose transporters (SGLTs), predominately SGLT2 (approximately 90%). Inhibition of SGLT2 results in increased levels of glucosuria and a corresponding reduction of blood glucose levels (Ghosh *et al*, 2012; Misra, 2012). Dapagliflozin is a selective SGLT2 inhibitor now licensed for use in the UK and is expected to be issued by June 2013. (http://www.nice.org.uk/guidance/index)

1.16 Diet

Before the discovery of exogenously administerable insulin in the early twentieth century by Banting and Best, low carbohydrate 'starvation diets' were prescribed to reduce glycosuria in diabetic patients. Although these delayed death by preventing hypoglycemic coma most patients ultimately died of starvation (Sawyer and Gale, 2009). Various diets have since been recommended for T1DM in conjunction with insulin therapy including high

carbohydrate, normal diet and high fibre diet (Sawyer and Gale, 2009). T2DM has been associated with obesity and therefore frequently with a calorie dense diet and a sedentary lifestyle (Torpy *et al*, 2009). Various diets have also been recommended from various sources to counteract the current obesity and T2DM epidemic including the pre insulin diets recycled and modified as the Atkins diet (Sawyer and Gale, 2009). Starvation diets recently attracted considerable new attention for treatment of T2DM but larger studies are required to determine what proportion of T2DM patients will be able to use this as a practical treatment (Lim *et al*, 2011).

1.17 Searching for novel plant compounds with utility in T2DM

As already discussed, metformin, salicylate and aspirin, all originally derived from plants, exhibit efficacy in treatment of T2DM in humans. Other plant compounds are under investigation. As T2DM is largely attributable to the overconsumption of energy rich food, if healthy, palatable medicinal foods could be formulated, it may be possible to reverse the current trend of increasing obesity and T2DM. T2DM is a particularly promising target for this type of approach because it often precedes many other health problems which might be prevented by more effective treatment of T2DM. Plant compounds under investigation include theaflavins, polyphenolic compounds found in black tea extract which have already been proposed as potential antihyperglycemic agents (Cameron *et al*, 2008). Polyphenols have also been proposed to be beneficial in other conditions that T2DM may precede including Alzheimer's Disease (Pasinetti and Ho, 2010), cancer (He *et al*, 2008) and cardiovascular disease (Mukamal, 2002).

The ‘French paradox’ is the observation that French people suffer from a comparatively low incidence of cardiovascular pathology compared to the rest of Europe despite having a relatively high fat diet which would normally be believed to contribute to heart disease (Ferrieres, 2004). The mechanism and even existence of this effect has been contested but one possible and popular explanation has been that the French consume more red wine, containing phenolic compounds which may have antioxidant effects which could attenuate the oxidation of low density lipoproteins (LDL) and which have also been proposed to have anti-inflammatory properties which may be beneficial to the endothelium (Kappagoda *et al*, 2000). However flawed this simple connection may be, the link between intake of dietary phenolics (especially from fruit and vegetables) and health benefits has gathered momentum (Seeram, 2012). Therefore in this thesis, a variety of polyphenolic and other plant compounds have been investigated for therapeutic properties relevant to T2DM.

1.18 Aims and objectives

The aims of this project were to:

1. To extract polyphenolic compounds from grape seed, pine bark and berries using chromatographic techniques and to characterise these compounds using liquid chromatography/ mass spectrometry (LCMS).

2. To exclude or confirm insulin-like or metformin-like properties of these compounds by application to cell culture models to assess effects on regulators of energy homeostasis by measuring phosphorylation of the transcription factor FOXO1a, AMPK and the ribosomal protein S6.

CHAPTER 2 – MATERIALS AND METHODS

2.1 Materials

2.1.1 Lysis buffer

This consisted of 50mM Tris acetate adjusted to pH 7.5 with acetic acid, 0.27M sucrose (Fisher Scientific), 1mM ethylenediaminetetraacetic acid (EDTA, Fisher Scientific), 1 mM ethyleneglycoltetracetic acid (EGTA, Tocris Bioscience), 100 mM sodium orthovanadate (Sigma- Aldrich), 10 mM β-glycerophosphate (Sigma), 50mM sodium fluoride, 5mM sodium pyrophosphate and 1% Triton X-100 (BDH; w/v 1.06g/ ml). 10µl β-mercaptoethanol (Sigma,), 10µl phenylmethanesulphonylfluoride (PMSF, Sigma,) and 100µl 0.1M sodium orthovanadate were added to 10ml lysis buffer before use as their activity rapidly diminishes. Sucrose facilitates osmotic burst as when it is taken up by the cell, water osmotically follows. EDTA is a metal ion chelator and therefore acts as a metal ion dependent protease inhibitor. EGTA has a higher affinity for calcium ions than other ions and is used to inhibit calcium dependent phosphatases and protein kinases. Sodium orthovanadate inhibits protein tyrosine phosphatases which could otherwise remove phosphate groups from phosphorylated tyrosine residues on proteins. β- glycerophosphate and sodium fluoride are serine/threonine phosphatase inhibitors and sodium pyrophosphate is a general phosphatase inhibitor. Triton X-100 is used to permeabilise the cell membrane. β-mercaptoethanol was used to denature proteins to their secondary structure by cleaving disulphide bonds and stabilising the protein. The intermediate compound formed can then form a stable structure preventing further

reaction with the resultant free sulphydryl residues resulting in reformation of the bond. PMSF is a serine protease inhibitor.

2.1.2 Tris Buffered Saline X 10

2 litres (L) Tris Buffered Saline (TBS) X10 consisted of 584.4 grams (g) sodium chloride (NaCl); (Aldrich) and 48.5g Tris (Fisher Bioreagents,) made up to 2L using distilled water and mixed using a magnetic stirrer. Tris can buffer at the normal mammalian physiological pH of 7.4. The pH of this solution was brought down to 7.4 using concentrated hydrochloric acid (HCl) and thereafter bottled and autoclaved. This buffer is used in Western blotting to maintain pH with a stringent (0.5M) salt wash to reduce non-specific interactions. For blotting, 1X TBS was used, with addition of 0.1 % tween 20 (TBST).

Tween 20 (Polysorbate 20) is a polysorbate surfactant which can be used as a washing agent in immunoassays such as Western blots to prevent non specific antibody binding. Blocking buffer consisted of TBST with 5% milk w/v, mixed using a magnetic stirrer.

In western blotting, non-specific binding of proteins is prevented by placing the membrane in this dilute solution of milk. The proteins in the milk attach to the membrane in all places where the target proteins have not attached, termed ‘blocking’.

2.1.3 Transfer buffer X10

Transfer buffer is an electrolyte solution used to transfer proteins from a gel onto a nitrocellulose membrane. An electric current is passed through the

buffer which transfers the proteins from the gel onto the membrane. This buffer consisted of 288g glycine (Melford) and 60g Tris made up to 2L using distilled water, mixed using a magnetic stirrer and thereafter bottled and autoclaved. Before use, 300ml of this stock was added to 2400ml distilled water and 300ml methanol added.

2.1.4 MOPS (morpholino propanesulfonic acid) buffer X1

MOPS running buffer X 1 consisted of 950ml distilled water and 50ml X20 MOPS buffer (83.7g MOPS; 13.6g sodium acetate trihydrate; 3.7g EDTA disodium dehydrate, 800ml nuclease free distilled water, adjusted to pH 7), made up to 1L. This buffer allows a current to flow through the gel, enabling electrophoretic movement of the proteins loaded on the gel. 500µl NuPage antioxidant was added to 200ml MOPS X1 to prevent reoxidation of reduced disulphide bonds within the proteins of interest.

2.1.5 Loading buffer

This consisted of 10g sodium dodecyl sulphate (SDS), 12.5 ml 0.5M Tris-HCl pH 6.8 and 50ml glycerol (Sigma- Aldrich) made up to 100ml with 25mg bromophenol blue (Sigma- Aldrich). Before use the loading buffer was heated twice for ten seconds or until no crystallisation was observed at full power in a microwave oven and 5% β-mercaptoethanol added.

SDS is an anionic surfactant which disrupts non covalent bonds in the proteins, causing them to lose their secondary and tertiary conformation. SDS also confers a negative charge on the protein at a ratio of one SDS

anion for every 2 amino acid residues, which is therefore proportional to the mass of the protein (Biological Industries, 2012).

2.1.6 Grape seed and pine bark extracts

Masquelier's grape seed extract and pine bark extract were obtained separately from Berkem. They are commercially available as a grape seed and pine bark oligopranthocyanidin homeopathic mixture and sold to the public in tablet form.

2.1.7 Commonly used compounds.

Commonly used compounds are listed in Table 2.1:

Compound	**Supplier**
Tris base	Fisher, Loughburgh, LE11 SRG
Acetic acid	VWR, Leicestershire, LE17 4XN
EDTA	Gibco, Paisley, PA4 9RF
EGTA	Tocris Bioscience, Bristol BS11 OQL
Sodium orthovanadate	Sigma- Aldrich, Dorset, England
B-glycerophosphate	Sigma, Dorset, England
Sodium fluoride	Fischer Scientific, Loughburgh, LE11 SRG
Sodium pyrophosphate	BDH, Hull, HU1 1UY
Triton-X	Sigma, Dorset, England
B-mercaptoethanol	Aldrich, Dorset, England
PMSF	Sigma, Dorset, England
Sodium chloride	Aldrich, Dorset, England
Tween 20	Sigma, Dorset, England
Glycine	Sigma, Dorset, England
Methanol	Fisher chemical, Loughburgh, LE11 SRG
SDS	BDH, Hull, HU1 1UY
Hydrochloric acid	BDH, Hull, HU1 1UY
Glycerol	Sigma- Aldrich, Dorset, England
Bromophenol blue	Sigma- Aldrich, Dorset, England
Dubelco's Modified Eagle Medium	Gibco, Paisley, PA4 9RF
Fetal Bovine Serum	Gibco, Paisley, PA4 9RF
SeeBlue Plus2 Prestained standard	Invitrogen, Paisley, PA4 9RF
Trypsin	Gibco, Paisley, PA4 9RF

Table 2.1: Synopsis of commonly used chemicals

2.1.8 Primary and secondary antibodies.

Some commonly used primary and secondary antibodies are shown in Table 2.2

Primary	**Supplier**	**Secondary**
FoxO1a	NG Rena, University of Dundee	sheep
pSer325 FoxO1a	NG Rena, University of Dundee	sheep
PKB Ser 473	Cell Signaling Technology, Hertfordshire, SG4 OTY	rabbit
ACC	Cell Signaling Technology, Hertfordshire, SG4 OTY	rabbit
S6	Cell Signaling Technology, Hertfordshire, SG4 OTY	rabbit
pACC Ser 79	Division of Signal Transduction Therapy, University of Dundee	sheep
pS6 Ser 240/244	Cell Signaling Technology, Hertfordshire, SG4 OTY	rabbit
Actin	Calbiochem, Irvine, KA12 8NB	mouse

Table 2.2: Some commonly used antibodies

2.1.9 Materials for extraction and analytical studies

The suppliers for materials used for extraction and analysis of samples are shown in Table 2.3:

Compound	**Supplier**
Bradford Reagent	Sigma, Dorset, England
Folin Ciocalteu Reagent	Sigma, Dorset, England
Sodium carbonate	Fluka, Irvine, KA12 8NB
Sodium acetate	Fluka, Irvine, KA12 8NB
Hydrochloric acid	BDH, Hull, HU1 1UY
Potassium chloride	BDH, Hull, HU1 1UY
Ferric ion	BDH, Hull, HU1 1UY
Tripyridyltriazine	Sigma, Dorset, England
Methanol	Fisher Chemical, Loughburgh, LE11 SRG
Acetone	Fisher Chemical
Ethyl acetate	BDH, Hull, HU1 1UY
Trichloromethane	BDH, Hull, HU1 1UY
Ethanol	Fisher Chemical
Diethyl ether	Sigma-Aldrich, Dorset, England
Sephadex LH column	GE Healthcare, Buckinghamshire, HP8 4SP
Supelco Discovery Polyamide DPA-6S cartidges	Supelco, Dorset, England
Synergi HPLC column 4μM Hydro- RP 80A RP C18 150 x 4.6mm	Phenomenex, Cheshire, SK10 2BN
Formic acid	Fluka, Irvine, KA12 8NB
HPLC grade acetonitrile	Sigma-Aldrich Dorset, England
HPLC grade dichloromethane	Sigma-Aldrich, Dorset, England
HPLC grade methanol	Sigma-Aldrich, Dorset, England

Table 2.3: Commonly used reagents for extraction and analysis of samples.

2.1.10 Benzoic acids and their analogues

The suppliers of commonly used chemicals for benzoic acid studies are shown in Table 2.4:

Compound	**Supplier**
Benzoic acid	Sigma, Dorset, England
2-hydroxybenzoic acid	Sigma- Aldrich, Dorset, England
3-hydroxybenzoic acid	Aldrich, Dorset, England
4-hydroxybenzoic acid	Aldrich, Dorset, England
2,3-dihydroxybenzoic acid	Aldrich, Dorset, England
2,4-dihydroxybenzoic acid	Aldrich, Dorset, England
2,5-dihydroxybenzoic acid	Aldrich, Dorset, England
2,6-dihydroxybenzoic acid	Aldrich, Dorset, England
3,4-dihydroxybenzoic acid	Aldrich, Dorset, England
3,5-dihydroxybenzoic acid	Aldrich, Dorset, England
3,4,5-trihydroxybenzoic acid	Sigma, Dorset, England
3,5-dimethoxy- 4- hydroxybenzoic acid	Aldrich, Dorset, England
p-anisic acid	Aldrich, Dorset, England
2-methoxybenzoic acid	Aldrich, Dorset, England
4-ethylbenzoic acid	Aldrich, Dorset, England
3-hydroxy-4-methoxybenzoic acid	Aldrich, Dorset, England
4-hydroxy-3-methoxybenzoic acid	Aldrich, Dorset, England
4-hydroxybenzaldehyde	Aldrich, Dorset, England

Table 2.4: Some commonly used chemicals in benzoic acid studies.

2.1.11 Diguanide synthesis

Suppliers of some commonly used chemicals for diguanide synthesis are shown in Table 2:5

Compound	**Supplier**
Methylisourea	Sigma- Aldrich, Dorset, England
Potassium hydroxide	BDH, Hull, HU1 1UY
Cadaverine	Sigma- Aldrich, Dorset, England
Perichloric acid	BDH, Hull, HU1 1UY
1,7-diaminoheptane	Sigma- Aldrich, Dorset, England
1,8-diaminoheptane	Sigma- Aldrich, Dorset, England
Spermine	Sigma- Aldrich, Dorset, England
Spermidine	Sigma- Aldrich, Dorset, England
Arcaine	Tocris Bioscience, Bristol BS11 OQL
1,6- Bis(guanidine) hexane sulfate	Santa Cruz, Middlesex, HA9 7YN
1-Dodecyl-guanidiniumacetat	Fluka, Irvine, KA12 8NB

Table 2.5: Sources of commonly used chemicals for diguanide synthesis

2.1.12 Gene expression reporter and WST-1 assay reagents

The sources of some commonly used reagents for gene expression reporter assays and for WST-1 assays are shown in Table 2.6:

Compound	**Supplier**
Dexamethasone	Sigma, Dorset, England
Cyclic adenosine monophosphate (cAMP)	Calbiochem, Irvine, KA12 8NB
Insulin	Novo Nordisk, West Sussex, RH11 9RT
Metformin	Calbiochem, Irvine, KA12 8NB,
Luciferase assay reagent	Promega, Southampton, SO16 7NS
Luciferase assay lysis buffer	Promega, Southampton, SO16 7NS
WST-1 assay	Millipore, Dundee DD2 1SW

Table 2.6: Sources for commonly used reagents for gene expression and WST-1 assays

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Maintenance

Cell culture is a technique whereby cells are grown under controlled conditions. All cell culture techniques were carried out under aseptic conditions and all media was heated to 37^{o}C before use. Cells were cultured at 37^{o}C in 75cm^{3} flasks (Corning) in a 5% carbon dioxide (CO_2) water saturated incubator.

2.2.1.2 Human Embryonic kidney 293 cells

Human embryonic kidney (HEK) 293 cells were used as they express a high and therefore detectable level of FOXO1a. These cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) containing 1g/l glucose, L-glutamate and pyruvate supplemented with 10% (v/v) foetal bovine serum (FBS, GIBCO). The cells were passaged three times a week (1:4). Passage of cells was achieved by aspirating the cell culture medium and washing twice with 5ml sterile DMEM before the cells were dissociated from the flask by the addition of 5ml 0.25% trypsin. The cells were allowed to detach for one minute at 37^{o}C in a 5% CO_2 water saturated incubator before inactivation of trypsin by the addition of 50ml DMEM supplemented with 10% FBS. 10ml of this culture was transferred to a new sterile 80cm^{2} flask to maintain stocks and the remainder divided between ten 6cm^{2} dishes for further experimentation.

2.2.1.3 H4IIE cells

This cell line was used to detect phosphorylated ACC (pACC), phosphorylated AMPK (pAMPK) and phosphorylated S6 (pS6). They are rat hepatocytes where gluconeogenesis is regulated by insulin in a similar way to human liver cells. Cells were maintained as above except 5% FBS was used and a 1:4 passage was carried out twice weekly. The cell line was a generous gift from Dr Calum Sutherland.

2.2.2 Cell stimulation and lysis

Four days after passage, DMEM with FBS was renewed 24 hours before experimentation and prior to incubation with stimuli, the cells were serum starved for 30 minutes by rinsing the cells once in 4ml DMEM, which was replaced by 2ml DMEM. Each plate was then treated and labelled as required and incubated at 37^{o}C in a 5% CO_2 water saturated atmosphere for one hour. The DMEM was removed from the plates which were then placed on ice and lysis buffer added, the cells scraped into the buffer and the buffer/protein mixture placed into correspondingly labelled eppendorf tubes which were centrifuged at 13,000 rpm for five minutes at 4^{o}C. 150µl supernatant was removed then placed into labelled eppendorf tubes and the tubes containing the pellets were stored at -80^{o}C. Loading buffer was then heated 2X10 seconds at full power in a microwave oven or until no SDS crystallisation was observed and 5% β-mercaptoethanol added. 38µl loading buffer was then added to each of the tubes containing 150µl supernatant which were then vortexed and stored at -20^{o}C.

2.2.3 Cell reporter assay

G6Pase expression was measured using a luciferase cell reporter assay. Luciferase and luciferin are endogenous to the firefly. Luciferin reacts with oxygen, ATP and luciferase to produce light. LLHG (Lisa Logie human G6Pase) cells are H4IIE cells transfected with a G6Pase/ luciferase DNA construct. In this construct, luciferase expression is under the control of the human G6Pase promoter, so luciferase expression monitors the activity of the promoter. G6Pase expression is induced by glucocorticoids such as dexamethasone and by glucagon via cAMP. Therefore combined dexamethasone (100μM) and cAMP (500nM) was used as a positive control to induce G6Pase expression and the negative control involved suppression by treatment with 10nM insulin.

2.2.4 WST1 assay

A WST1 assay is a colorometric measurement of cell viability. It is based on the cleavage of the tetrazoium salt WST1 to formazin by succinate tetrazolium reductase, a mitochondrial dehydrogenase resulting in a colour change from a pale to a darker yellow, depending on the number of viable cells present. Water soluble tetrazolium salts were developed by introducing positive or negative charges and hydroxyl groups to the phenyl ring of the tetrazolium salt. The bioreduction of WST1 is thought to depend on glycolytic NADPH production and therefore reports the presence of viable cells.

H4IIE cells were seeded on a 96 well plate, starved and treated with the appropriate samples. WST1 mixture was added to each well, mixed and

incubated for two hours. The absorbance of each sample was measured using a spectrophotometer at a wavelength of 250nm.

2.2.5 Western blotting

Western blotting is an analytical technique used to detect specific proteins in a sample after separation of the proteins by SDS-PAGE.

2.2.5.1 SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis)

Samples in loading buffer were removed from -20^{o}C storage, thawed and heated at 95^{o}C for 5 minutes on a heating block to denature the proteins. The proteins in each sample were separated by SDS-PAGE on NuPage 4-12% Bis-Tris gels (Invitrogen). The reservoirs of the electrophoresis apparatus were filled with 1X MOPS running buffer and the central reservoir also contained a 1/400 v/v concentration of NuPage antioxidant (Invitrogen). The wells of the gels were then rinsed with running buffer and loaded with 1µl SeeBlue Plus2 Prestained Standard (Invitrogen) or 15µl sample as required. The gels were then run for 50 minutes at 200V when probing for FOXO proteins and 2hrs at 90V when probing for ACC, AMPK and S6 proteins, as this allowed better resolution of ACC. The remainder of each sample was stored at -20^{o}C.

2.2.5.2 Protein Transfer

Once the proteins had been separated, they were electroblotted to transfer them onto nitrocellulose membranes. The gel was placed into a transfer

cassette as follows. A blotting pad was placed into the transfer cassette followed by two pieces of blotting paper and a nitrocellulose membrane (Whatman) then the gel was placed on top followed by another two pieces of blotting paper and a blotting pad. After ensuring no air bubbles remained within the cassette, the cassette was closed and loaded into transfer apparatus which was then filled with transfer buffer and run overnight at 0.12A, over two days at 0.1A, three days at 0.07A or four days at 0.05A current. Once the proteins had transferred, the nitrocellulose membranes were removed from the cassette and were stained with Ponceau (Invitrogen) to confirm that the protein content of each of the samples was equivalent. The membranes were then rinsed briefly three times with TBST to rinse off the Ponceau stain, renewing the TBST after each rinse.

2.2.5.3 Immunoblotting and exposure

The membranes were then blocked with TBST + 5% milk and placed on a platform rocker for one hour. Then the blot was treated with the specific primary antibodies in 10ml TBST with 5% milk and incubated overnight at 4^{o}C. The membranes were then quickly rinsed in TBST three times then rocked in TBST for ten minutes followed by two five minute periods with fresh TBST after each rinse or wash. The blots were then incubated with the secondary antibody specific to the primary antibody host for 1 hour in 10ml TBST + 5% milk The secondary antibody was coupled to horse radish peroxidase (HRP) to enable detection by chemiluminscence. After incubation in secondary antibody, the blots were quickly rinsed in TBST three times and rocked with TBST for ten minutes, rinsed and rocked with TBST again for a

further two five minute periods. The blots had excess buffer removed, were placed in a clean dish and 2ml of enhanced chemoluminescence (ECL) reagent (Amersham Biosciences) was added for one minute. The blots were removed, the excess reagent removed and the blot transferred to a film cassette lined with cling film. In the dark room, typically one, five fifteen minute and overnight exposures were captured on X-ray film (Fuji), carried out under a safe light.

2.2.6 Bradford assay

This method measures the concentration of protein in a sample (Bradford, 1976). When proteins bind to Coomassie Brilliant Blue G-250 (Bradford reagent, Sigma), the dye changes from red to blue causing a change in absorption from 465nm to 595nm. A standard curve was generated using incrementally increasing concentrations of protein standard and the protein concentration of each sample was determined using this curve.

$y = mx + c$

$x = y – c/m$ where:

x = protein concentration

y = absorbance at 595nm

m = gradient

c = y intercept

2.2.7 Total phenols assay

All assays were carried out in triplicate to determine the phenolic content of each sample. Samples were diluted to 1% in distilled water. 250µl half

strength Folin Ciocalteu Reagent (Sigma F295 with distilled water) was added to 250µl of each sample. After three minutes, 500µl of saturated sodium carbonate solution was added and one hour later, the absorbance of the sample at 750 nm was measured using an Ultrospec 2100 pro spectrophotometer. Reduction of molybdenum in the Folin Ciocalteu Reagent results in a colour change from yellow to blue under the basic conditions provided by sodium carbonate. Five incrementally-increasing concentrations of phloroglucinol were used to generate a standard curve. The values obtained were then analysed on an Excel programme using the following equation:

$y = mx + c$; therefore: $x = y/m - c$; $c = 0$ therefore: $x = y/m$ where:

y = phenolic concentration in diluted sample (µg);

x = absorbance of sample at λ 750nm

m = gradient

The result obtained was then multiplied by 100 to give the phenolic concentration of 100% sample.

2.2.8 Total anthocyanin assay

The total anthocyanin assays were performed using a pH differential method. At pH1, anthocyanins exist predominately in the form of the red flavylium cation and increasing the pH to 4.5 leads to a decrease in the concentration of the flavylium cation and therefore a decrease in colour intensity. Anthocyanins absorb well at 510 nm at pH 1 and poorly at pH 4.5. The measurements at 700 nm pick up compounds that are brown at these pHs and interfere with the measurement at 510 nm. The absorbance correlates

with the concentration of anthocyanins in samples according to the follows the Beer Lambert law.

$(A_{510} - A_{700})_{pH1} - (A_{510} - A_{700})_{pH4.5}$

Samples were diluted to 2% final concentration (20µl sample/ 980µl distilled water) in both 0.2M HCl/ KCl pH1 and 0.1M sodium acetate pH 4.5 (2 repeats per sample). A blank of 0.2M HCl /KCl pH1 was used. After a 15 minute incubation, the absorbance of each sample was measured on a spectrophotometer at wavelengths of 510 and 700 nm.

The values obtained were then entered into an Excel programme using the above equation. All assays were carried out in duplicate and the average values used to estimate the anthocyanin content of each sample.

2.2.9 Ferric reducing antioxidant power (FRAP) Assay

Each sample was diluted to 1% in distilled water. A ferric complex was made from ferric ion (Fe^{3+}) and tripyndylthiazine (TPTZ) and the degree to which samples reduced this to the ferrous form (Fe^{2+}), changing its colour to blue, was measured at a wavelength of 593nm over 4 minutes. A standard curve is generated using known concentrations of ferrous ion using ferrous sulphate. The values obtained were entered into an Excel programme using the following equation:

y = mx + c; therefore: x = y/m – c; c = 0 therefore: x =y/m where:

x = FRAP

y = absorbance of sample at λ593nm

m = gradient

2.2.10 Chromatography

This is a technique whereby compounds within a mixture are separated according to their physical and/ or chemical properties. Preparative chromatography involves purification of a sample and analytical chromatography involves measuring the relative proportions of analytes in a sample.

Chromatographic techniques can be classified according to the state of either the mobile or the stationary phase, by the separation mechanism involved, by the comparative polarities of the mobile and stationary phases or by size.

In gas chromatography the mobile phase is a gas and the stationary phase is a liquid set within a glass or metal column. In liquid chromatography the mobile phase is a liquid and the stationary phase is a solid.

In column chromatography the stationary phase is a solid support set within a tube and the mobile phase is liquid. In size exclusion chromatography molecules are separated according to size. Smaller molecules are able to enter pores within the stationary phase and remain there whereas larger molecules are excluded and eluted.

In normal phase chromatography, the stationary phase is more polar than the mobile phase. In reverse phase chromatography, the mobile phase is more polar than the stationary phase.

2.2.11 Extraction of phenols from berries

100g berries were liquidised in 400ml of a 50/ 50 methanol/ acetone mix and placed in 50ml centrifuge tubes. Ultrasound assisted extraction (5 X 2 mins) was then applied to aid extraction by breaking down the plant cell wall and tubes were centrifuged at 3000 rpm for 4 minutes and supernatant collected. The pellets were redissolved in 30ml of the acetone/methanol solvent and re-extracted. A further extraction of the pellet was carried out using 20 ml solvent before all 3 extracts were combined. A total phenol assay was then carried out to determine the phenol content of the sample.

2.2.12 Solvent/ solvent fractionation

This method was adapted from that described by Saucier *et al*, (2001), which involved fractionation based on the relative solubility of proanthocyanidins (PACs) of different sizes in different solvents. A flow diagram is shown in Figure 2.1.

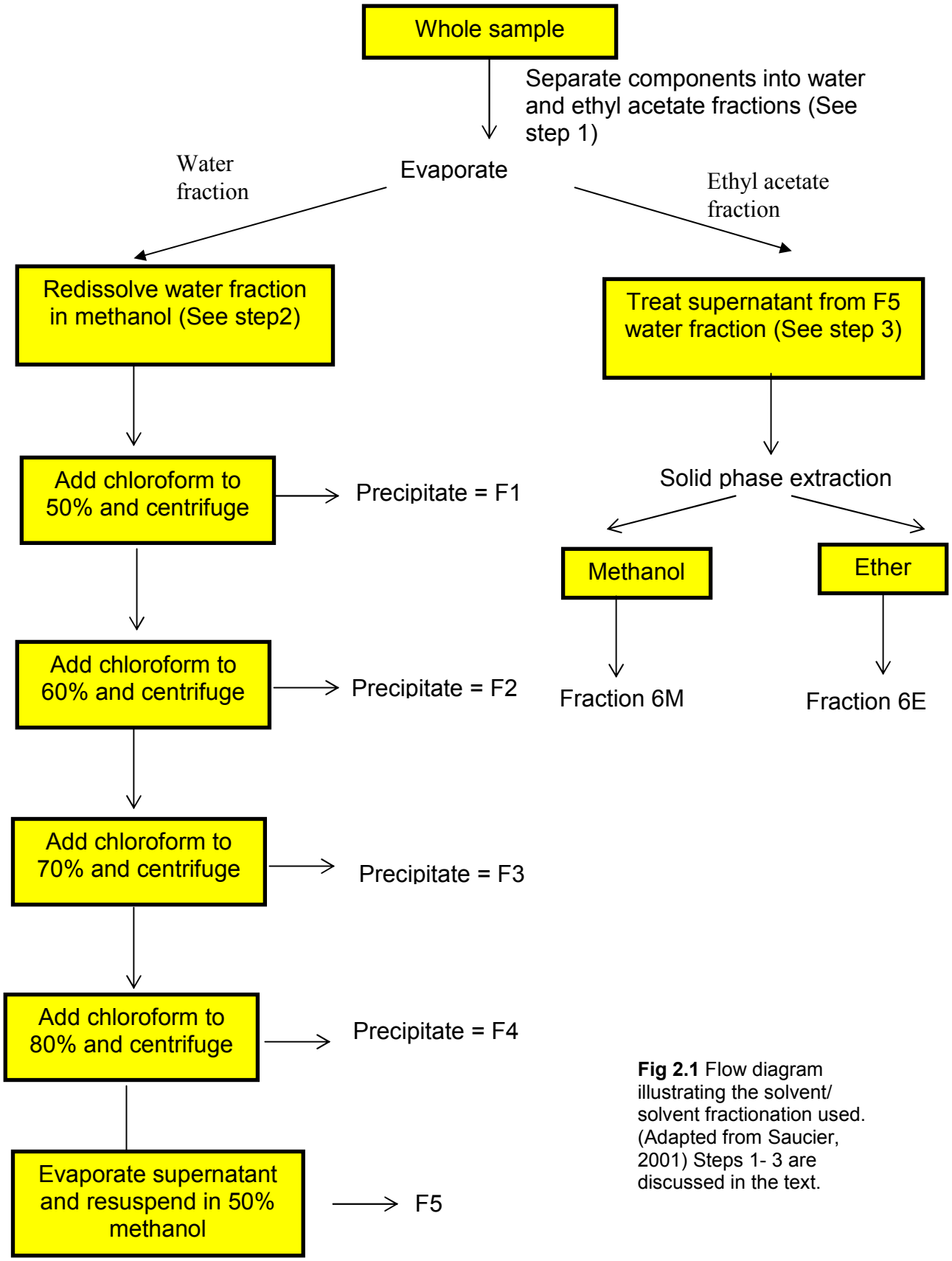


Fig 2.1 Flow diagram illustrating the solvent/ solvent fractionation used. (Adapted from Saucier, 2001) Steps 1- 3 are discussed in the text.

Step 1. Extraction of PACs in ethyl acetate

0.5g whole sample (GSE or PBE) was dissolved in 50ml distilled water and 20ml placed in a separating funnel. 20ml of ethyl acetate was added then the water and ethyl acetate fractions were separated into round bottomed flasks and dried on a rotatory evaporator.

Step 2. Treatment of ethyl acetate fraction

The water fraction was redissolved in 5ml methanol and 5ml chloroform added to raise the chloroform concentration to 50% (v/v). After mixing, the solution was centrifuged at 3000rpm for 10mins at 5^{o}C. The supernatant was removed and placed in a new tube and the pellet resuspended in 1ml methanol to generate Fraction 1 (F1). 2.5ml chloroform was then added to raise the chloroform concentration to 60% (v/v) and the sample was treated as before. The supernatant was removed and the pellet was resuspended in 1ml methanol to generate F2. 4.2ml chloroform was added to the supernatant to raise the chloroform concentration to 70% (v/v) and after centrifugation, the pellet was resuspended in 1ml methanol to form F3. 8.3ml chloroform was then added to the supernatant to raise the chloroform concentration to 80% (v/v). Again the pellet was resuspended in 1ml methanol to form F4 and the supernatant was labelled F5. Fractions 1- 4 were dried in the centrifugal evaporator and F5 in the rotatory evaporator.

Step 3. Solid phase extraction

The ethyl acetate fraction was then resuspended in 50% ethanol and a C18 SPE column (Thermo Scientific) was then prepared by filtering 35ml diethyl ether followed by 35ml methanol through the column. The sample was added and the run-through, including a 20ml distilled water wash, discarded. 50ml

diethyl ether was then added and an ether subfraction (F6) was collected. 50ml methanol was then added and a methanol subfraction of F6 was collected. 35ml distilled water was then added and a water subfraction of F6 was collected. These subfractions were then dried in the rotatory evaporator.

2.2.13 Column chromatography

2.2.13.1 Sephadex LH20 gel filtration column

0.5g (GSE or PBE) of sample was dissolved in 50ml 50% ethanol. A lipohilic Sephadex LH20 column (GE Healthcare) was then used to separate each sample into fractions largely according to hydrophobicity. The column was prepared according to the manufacturer's instructions and 5ml distilled water was passed through the column and discarded. The sample (total volume 5 ml) was applied to the column. The column was eluted with 50% ethanol and unbound sample collected. Bound components were eluted using 50% acetone. After this, 80% acetone was added and a final acetone wash sample collected. Then 20ml 50% ethanol was passed through the column and a final ethanol wash sample collected. Each fraction was assayed for total phenols and the following equation used to produce 250µg GAE aliquots:

250/ phenolic concentration in µg/ml) X 1000 = volume in no µl required to give 250µg GAE of sample

These samples were then dried in a centrifugal evaporator under vacuum.

2.2.13.2 Solid phase extraction on Discovery polyamide columns

This solid phase extraction (SPE) technique was adapted from a method described by Hellstrom *et al* (2007). SPE uses the affinity of molecules within a sample for a solid matrix through which the sample passes.

Supelco Discovery DPA-6S polyamide cartridges (Supelco) adsorb polar compounds such as proanthocyanidins through hydrogen bonding between amide groups on the column and hydroxyl groups on the proanthocyanidins were used. These units were set up on a SPE vacuum manifold. The column was first conditioned with 10ml 100% methanol and then 10ml distilled water. The sample (10ml) was placed on top of the bed and 10ml unbound wash (sample) was collected. Then 10ml distilled water was added and 10ml unbound wash collected. Elution with 10ml 20% methanol was used to elute the unbound components. After this, the bound components were eluted using 80% acetone. Finally, 10ml 100% methanol was added and a 10ml methanol wash collected and 10ml distilled water was added and 10ml water sample collected. Each fraction generated was aliquoted according to its phenol content and evaporated as previously described.

2.2.13.3 Liquid chromatography/ Mass spectrometry

Mass spectrometry (MS) is an analytical technique used to determine the molecular composition of a sample. The components of each sample and fraction generated were determined by liquid chromatography/mass spectrometry (LCMS) using an LCQ-Deca system which consisted of a surveyor autosampler, a chromatography column, a pump, a photodiode array (PDA) detector and a Thermofinnigan mass spectrometer. The

autosampler injects the sample onto the column and the pump creates a gradient of an aqueous /organic solvent mix through the column enabling separation of different components. The column is referred to as the stationary phase and the solvents as the mobile phase. The PDA detector detects the absorption of eluted components at different wavelengths. The mass spectrometer consists of an ion source, an electrospray ionisation (ESI) interface which ionized the samples (usually to a monovalent charge), a mass analyzer where the mass/ charge (*m/z*) ratio of components was detected. Mass fragmentation (MS^2) can then be used to further identify the ions through characteristic fragmentation of the parent ions.

Samples were prepared by dissolving 250µg phenol equivalents in 250µl solvent and vortexing. After centrifugation, the samples were pipetted into 100 µl inserts in autosampler vials. A hole was placed on top of each sample vial and the samples were loaded into the autosampler. The column was equilibrated prior to use in solvents A and B then pre-equilibrated at the starting %s.

2.2.13.4 Reverse phase high performance liquid chromatography

Reverse phase chromatography uses a non polar stationary phase and a polar mobile phase so the most polar molecules elute from the column first. A Synergi 4µm Hydro-RP 80A reversed phase C18 column, size 150mm X 4.6mm (Phenomenex), was initially used as this column has proved effective in separation of polyphenol components. The term C18 describes the hydrophobic alkyl chain length of the stationary phase. 4µm describes the particle size of the solid support, RP indicates reverse phase and 80A

indicates the average pore size within each particle. Ultra pure water (UPW) with 0.2% formic acid (FA) was used as the aqueous solvent A and 90% HPLC grade acetonitrile (ACN) containing 0.1 % FA as the organic solvent B. FA was used to improve peak resolution. A gradient of 5 – 40% solvent B was used over a 30 min run to separate components.

2.2.13.5 Normal phase HPLC

This was method adapted from Hellstrom *et al* (2007). Normal phase HPLC uses a polar stationary phase and a non polar mobile phase to separate components. The method used a Silica Luna 5µm 100A column. Dichloromethane:methanol:water (5:44:1) was used as the aqueous solvent and dichloromethane:methanol:water (42:7:1) as the organic solvent. The analytes have varying affinities for the stationary phase by non polar interactions so the order of elution differs from reversed phase HPLC.

2.2.14 Purity analysis

Purity was assessed using the peak area detection facility in XCalibur software. The area of the peak of interest was divided by the total area of peaks in the separation zone. The result was then multiplied by 100 to calculate the percentage purity value.

2.2.15 Diguanide synthesis

Diguanides were synthesised using a method adapted from Tricot, 1990 using 1 mole of a diamine and 2 moles of methylisourea. The methylisourea was added to ice cold UPW and the pH increased to 10 using 5M KOH. The

diamine was added and this was incubated for three hours, performing 15 minute checks to ensure that the pH was maintained at 10. The guanidinylated products are insoluble at pH 10 and precipitates formed were collected by centrifugation and washed twice with 10ml ice cold UPW by recentrifugation. The final precipitates collected were frozen at -20^{o}C and freeze dried. The purity of the products was assessed by direct injection MS analysis and MS^2 fragmentation was used (with 45% energy collision factor) to obtain further information on the products. More detailed information is included in chapter 6.

CHAPTER 3 – GRAPE SEED AND PINE BARK EXTRACTS

3.1 Introduction

Phenolic compounds consist of a hydroxyl group bound to a benzene ring. The simplest of these, phenol is illustrated in Fig 3.1:

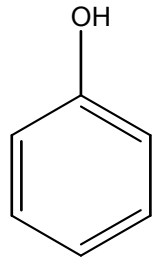


Fig 3.1: Structure of phenol

Polyphenols are compounds which contain many phenolic rings.

3.1.1 Flavonoids and proanthocyanidins

The flavonoids are a diverse sub-group of polyphenolic compounds based on a phenolic ring system. The basic flavonoid skeleton and associated numbering and lettering systems are illustrated in Fig 3.2:

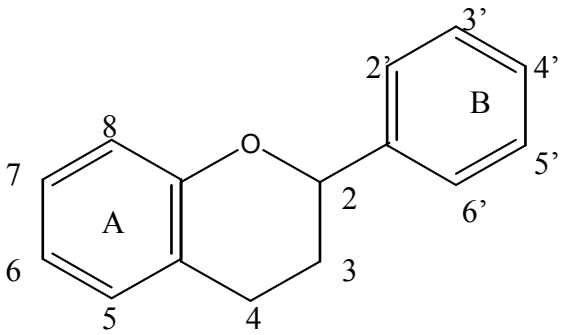


Fig 3.2 The basic flavonoid structure

Proanthocyanidins are polymerised flavonoids found in a wide variety of plant foods. They can exist as dimers up to polymers, with or without modification such as galloylation.

3.1.2 Benzoic acids

These consist of a benzene ring with an acid group. The simplest of these, benzoic acid, is illustrated in Fig 3.3:

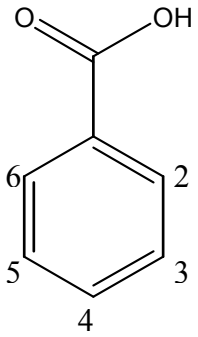


Fig 3.3: Benzoic acid.

3.1.3 Gallic acid and galloyl moiety

Gallic acid (3,4,5 trihydroxybenzoic acid) is a hydroxylated benzoic acid with an atomic mass of 170 atomic mass units (amu). Its structure is illustrated in Fig 3.4:

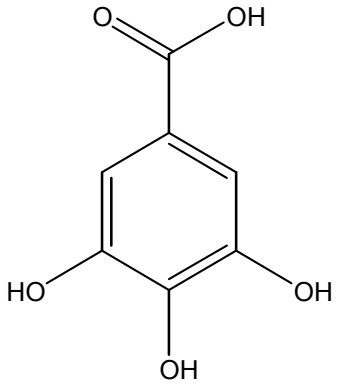


Fig 3.4: Gallic acid

Galloylation is a condensation reaction between the carboxylic acid group of gallic acid and a hydroxyl group of the PAC which results in the loss of a water molecule. This is illustrated in Fig 3.5:

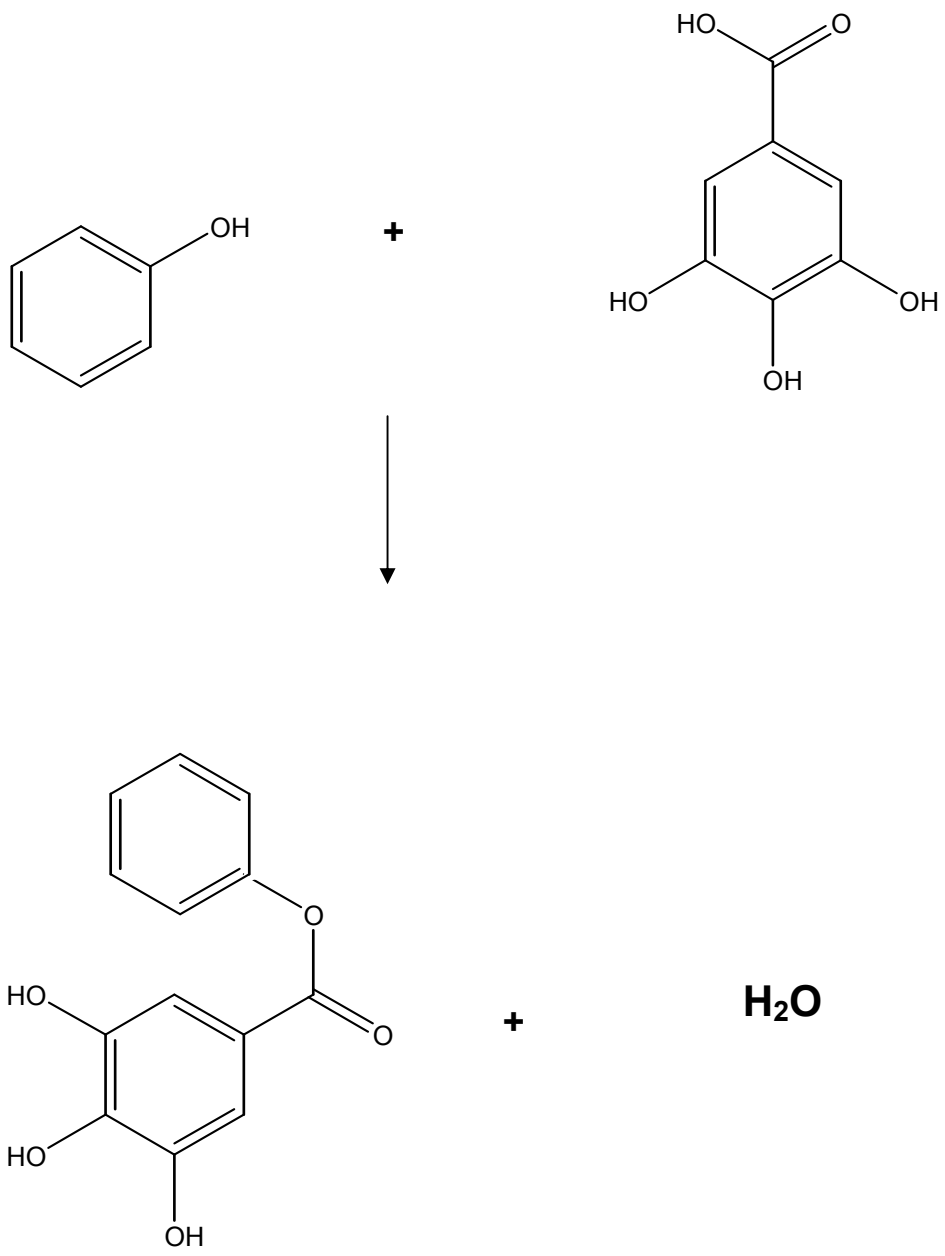


Fig 3.5: Reaction scheme of galloylation

A galloylated phenolic molecule is 152 amu heavier than its ungalloylated form i.e. 170 amu (gallic acid) – 18 amu (water) = 152 amu.

3.1.4 Grape seed and pine bark extracts

These are industrial products of grape seed and pine bark which have been shown to be rich in phenolic compounds (Weber *et al*, 2007). Plant compounds have provided a number of clinical and experimental therapies

for T2D and existing literature suggests polyphenols have a number of potential health benefits as described in Chapter 1. To search for insulin-like and metformin-like cellular responses to polyphenolic compounds, it was decided to fractionate grape seed and pine bark extracts and apply the fractions to cells. The purpose of this was to deduce chemical structural motifs or identify specific structures underlying any effects that were observed

3.2 Results

3.2.1 Insulin-like effects of grape seed and pine bark extracts

HEK293 cells were stimulated with incrementally increasing concentrations of grape seed extract (GSE) and pine bark extract (PBE) using no treatment (vehicle only) as a negative control and 50µg/ ml black tea extract (BTE) as a positive control. Western blotting confirmed that treatment with 100µg/ml GSE induced phosphorylation of FOXO1a. Similar but less pronounced induction was found for PBE. This is illustrated in Fig 3.6:

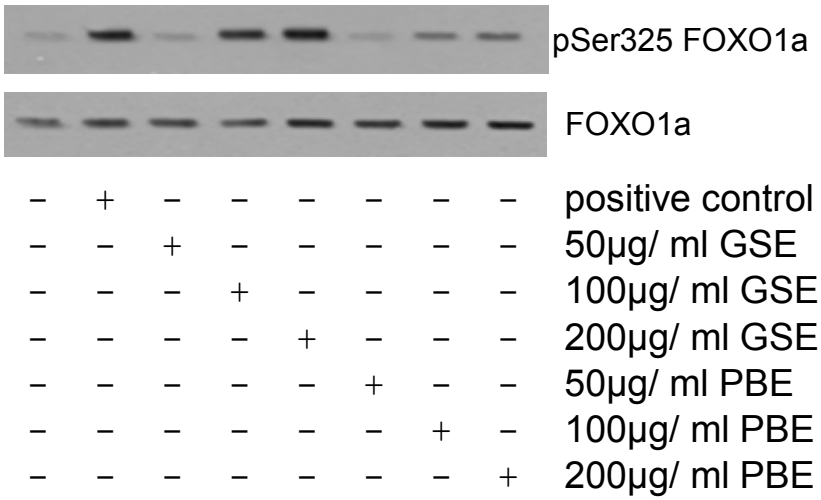
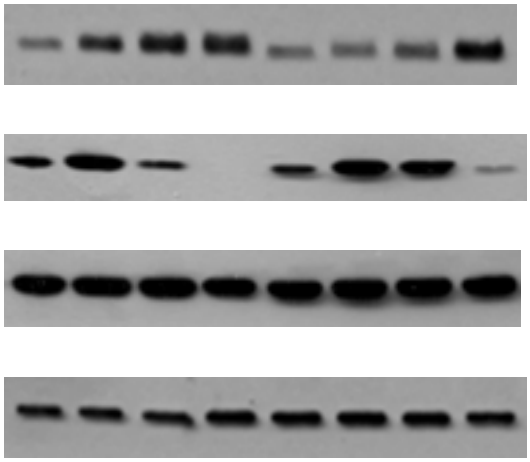


Fig 3.6: **Effect of GSE and PBE on FOXO1a phosphorylation.** Serum starved HEK293 cells were treated with incrementally increasing concentrations of GSE and PBE for one hour, followed by lysis and SDS-PAGE as described in the methods. Immunoblotting was carried out using an antibody that detects FOXO1a only if it is phosphorylated on Ser 325 (pSer325 FOXO1a) and a second antibody that detects FOXO1a regardless of phosphorylation state (FOXO1a). Negative control was DMEM only and positive control was 50µg/ ml Black Tea Extract (BTE)

3.2.2 Effect of GSE and PBE on AMPK and S6 phosphorylation

H4IIE cells were treated with incrementally increasing concentrations of GSE and PBE and 2 mM metformin was used as a positive control. Western blotting suggested that GSE was more effective than PBE in inducing phosphorylation. These results are shown in Fig 3.7:



−	+	−	−	−	−	−	−	50µg/ ml GSE
−	−	+	−	−	−	−	−	100µg/ ml GSE
−	−	−	+	−	−	−	−	200µg/ ml GSE
−	−	−	−	+	−	−	−	50µg/ ml PBE
−	−	−	−	−	+	−	−	100µg/ ml PBE
−	−	−	−	−	−	+	−	200µg/ ml PBE
−	−	−	−	−	−	−	+	positive control

Fig 3.7: Effect of GSE and PBE on AMPK and S6 phosphorylation.

Serum starved H4IIE cells were treated with incrementally increasing concentrations of GSE and PBE for three hours, followed by lysis and SDS-PAGE as described in the methods. Immunoblotting was carried out using an antibody that detects AMPK only if it is phosphorylated on Thr172 (pThr172 AMPK), an antibody that detects Ser 240/244 S6 phosphorylation (pSer240/244 S6) and an actin antibody. The negative control was DMEM only and the positive control 2mM metformin.

Similar to the differences in FOXO1a responses to GSE & PBE (Fig. 3.6), these results indicate that GSE was more effective than PBE at inducing AMPK phosphorylation (Fig. 3.7) and repressing S6 phosphorylation (Fig. 3.7). At low concentrations however, both GSE and PBE appeared to elevate S6 phosphorylation (Fig. 3.7).

3.2.3 Phenolic content of GSE and PBE.

A total phenols assay showed that GSE contained a higher concentration of phenolic compounds than PBE (Table 3.1).

Sample	Phenols (mg/ml)	SEM
GSE	6.3	0.1
PBE	4.7	0.1

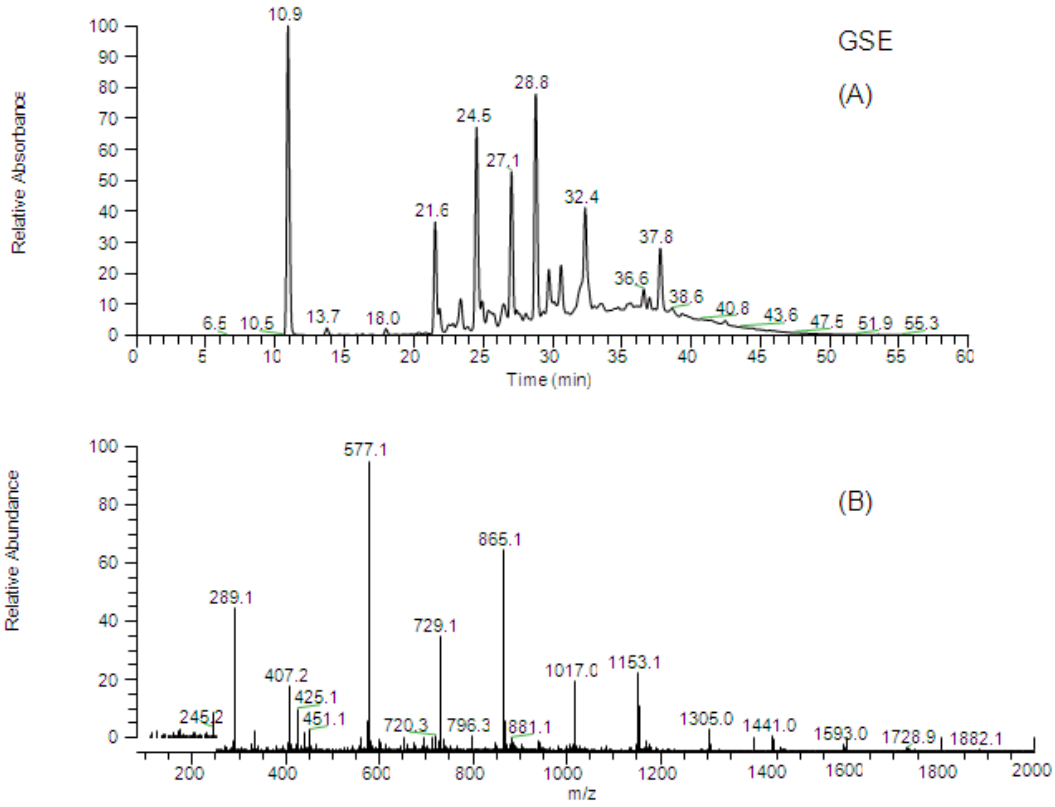
Table 3.1: Total Phenol content of GSE and PBE

Although the total phenol assays suggested a higher content of phenolic compounds in GSE than PBE, all subsequent cell culture based assays were carried out using equivalent phenol contents.

3.2.4 Analysis of phenolic compositon of GSE and PBE by LC-MS

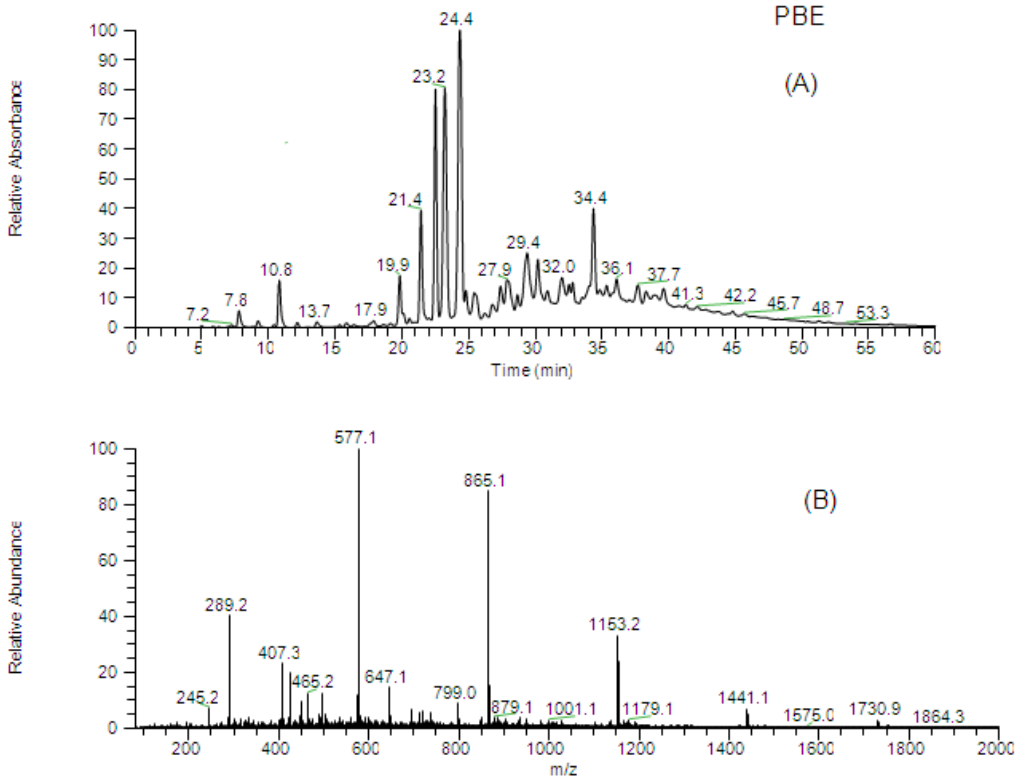
The composition of the GSE and PBE samples was analysed by LCMS. The components did not separate well using RP-HPLC and there was substantial overlap of peaks, particularly between 25- 40 mins (see Figs 3.8 and 3.9 A). Therefore the MS spectra obtained across the elution of the bound components (5-60 mins) was also used to indicate their phenolic composition (see Figs. 3.8 and 3.9 B). The MS data fitted with data from previous studies (Saucier *et al*, 2001; Hellstrom *et al,* 2008) on proanthocyanidin-rich extracts from GSE and PBE.

One obvious difference between the extracts was the presence of signals characteristic of galloylated PACs in GSE. This is highlighted in Fig 3.8 B, where peaks representing galloylated molecules that are 152 amu heavier than their ungalloylated counterparts are present in the GSE sample whereas they are absent in the PBE sample (Fig 3.9 B).



Retention time (mins)	Atomic weight (amu)	Putative ID
10.9	169.1	gallic acid
21.6	577.1	dimer
24.5	289.1	monomer
27.1	577.1	dimer
28.8	289.1	monomer
32.4	729.1	galloylated dimer
33- 35	1153.1 1305.0 1441.0	tetramer galloylated tetramer pentamer
36.6	577.1	dimer
37.8	1017.0	galloylated trimer
38- 55	1305.0 1593.0	galloylated tetramer galloylated pentamer

Fig 3.8: GSE chromatogram and MS. Highlighted peaks on GSE chromatogram scanned at λ280nm represent galloylated and ungalloylated PACs as detected by corresponding MS and listed in the table shown, beside putative IDs from Passos, 2007. Gallic acid is not well represented on the MS scan due to poor ionisation



Retention time (mins)	*m/z* [M-H]	Putative ID
10.8	169.1	gallic acid
19.9	371.1	caffeic acid derivative
21.4	577.1	dimer
22.5	495.1	lignan derivative
23.2	577.1	dimer
24.4	865.1	trimer
24.9	865.1	trimer
29.4	865.1	trimer
34.0	865.1	trimer
34.4	465.1	lignan derivative
36.1	1153.1	tetramer*
30-55	1153.1 1441.1 1730.0	tetramer* pentamer* hexamer*

Fig 3.9: Chromatogram and MS spectra from PBE. A= HPLC chromatograph at 280 nm; B = MS spectrum from 5-60 mins. The table gives putative peak identifications based on Karonen et al., 2004. The figures in the top right corner represent the FSD of the detectors. * - These PAC signals are in the unresolved “hump” of material.

It was also noted from this data that GSE contains a higher concentration of gallic acid (GA; RT = 10.9) than PBE and that PBE contained a substantial amount of non-PAC components including lignans. Therefore, although PBE and GSE were applied at the same concentration of total phenols, PBE contained less proanthocyanidins.

3.2.5 Fractionation of extracts by solvent to solvent partitioning

Fractionation of these samples was then carried out using the solvent to solvent partition method described in chapter 2, which has been used to produce fractions that differ in molecular weight. Briefly, fractionation was carried out on the following basis (Saucier *et al*, 2001).

Molecules of low degree of polymerisation (DP) should partition from a PAC sample dissolved in UPW into the ethyl acetate fraction, whilst those of higher DP should remain in the water fraction. Further fractionation of the ethyl acetate sample using methanol and diethyl ether may separate this fraction into molecules of larger (6E) and smaller (6M) DP. The water fraction, which should contain the higher molecular weight molecules, can be sequentially fractionated by their relative solubility in increasing concentrations of chloroform (i.e. fractions F1 to F4).

The chromatograms for each fraction are shown in Fig 3.10 and MS data in Fig 3.11. For identifications, we refer back to the Tables in Figs. 3.8 and 3.9. The corresponding PBE chromatography is shown in Fig 3.12 and the mass spectra in Fig 3.13.

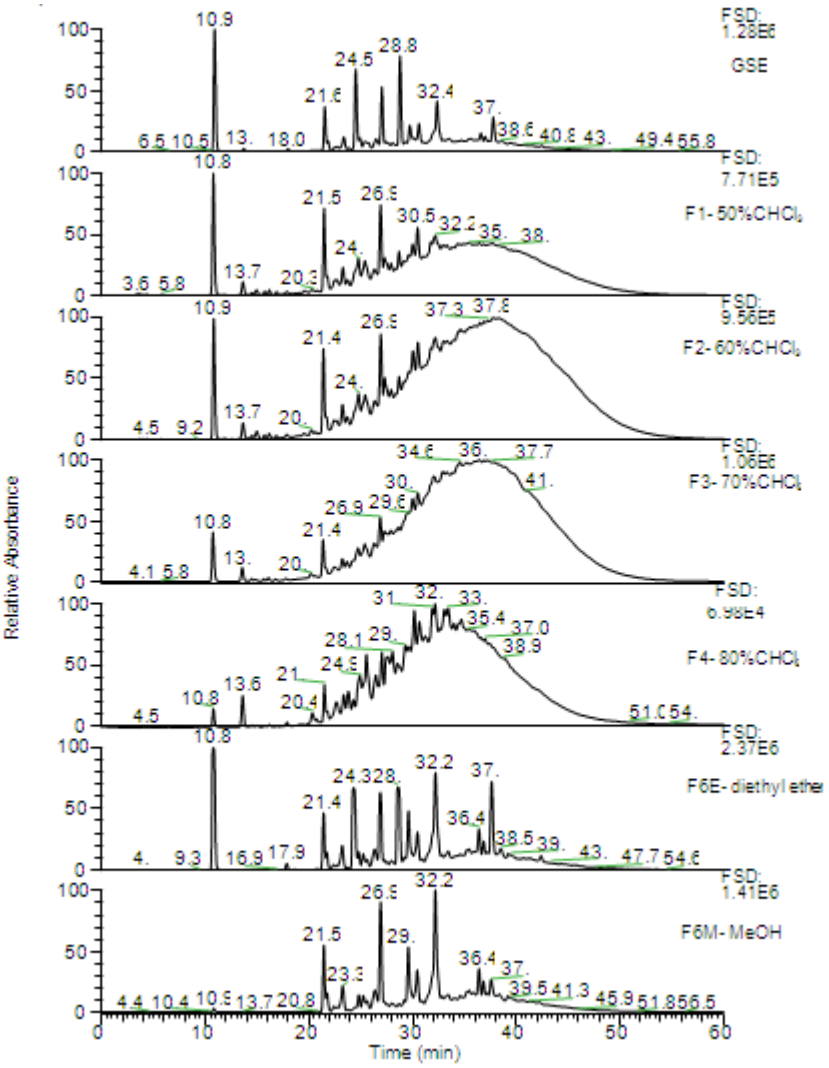


Fig 3.10: LC traces of GSE fractions obtained by solvent-to-solvent fractionation. Traces at 280 nm are shown. FSDs are shown in the top right corners of each trace.

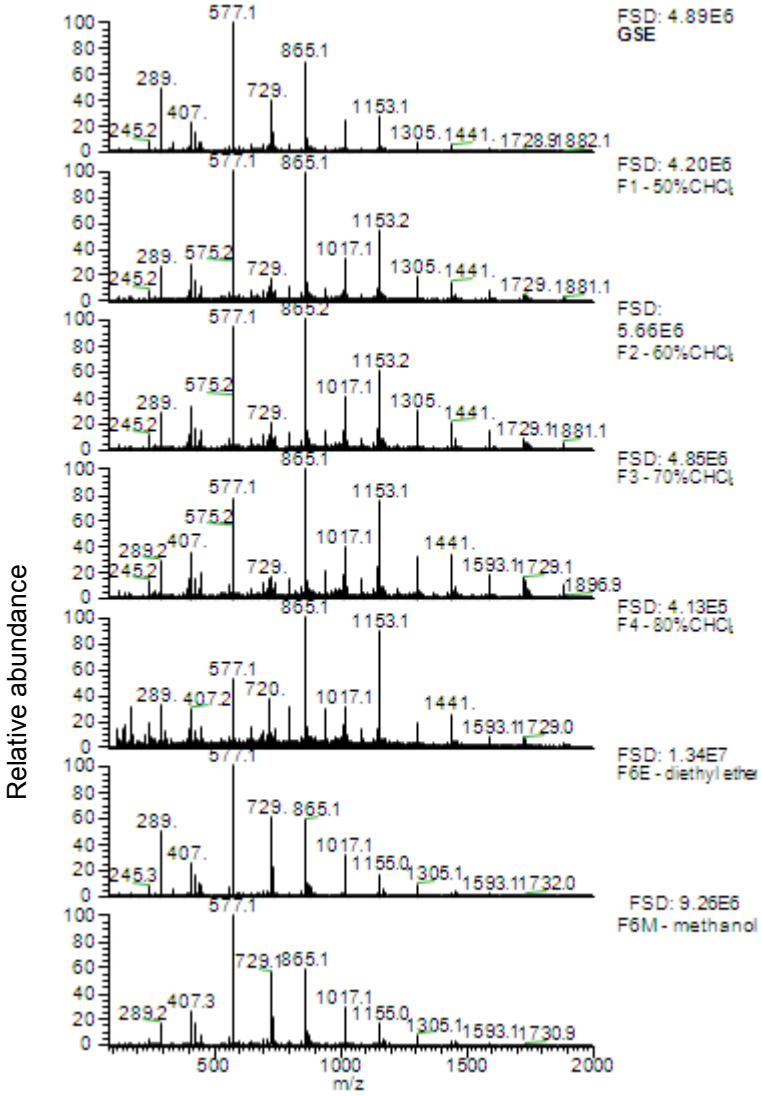


Fig 3.11: MS spectra of GSE fractions obtained by solvent-to-solvent fractionation. The FSD are shown in the top right hand corner of each panel.

The solvent-solvent partition of GSE largely followed the predicted pattern. The initial ethyl acetate fraction (which gives rise to fractions F6M and 6E) was enriched in low MW components including gallic acid (RT = 10.9), monomers (RTs 24.3 and 28.6), dimers (RTs 21.4 & 26.9) and some galloylated PACs (RTs = 32.2 & 37.6). There was some further fractionation of these peaks between fractions 6E and 6M. This generally fits with the MS spectra for fractions 6E and 6M (Fig. 3.11). In the water fraction (which ultimately yielded fraction F1- 4) there was a relative enrichment in higher MW PACs as judged by the PAC-rich hump (Fig. 3.10). There was also a relative enrichment of peaks at RT = 21.5 and 26.9 (dimers) in fractions F1 and F4, which accounts for their high intensity of *m/z* 577 signals (Fig. 3.11). Overall, there was a trend to enrichment in higher MW PACs through fractions F1 to F4 which was evidenced by the increasing relative intensity of *m/z* 1153 signals and the corresponding reduction in the intensity of the *m/z* 577 signal.

There was a similar pattern for the fractions obtained from the partition of PBE (Fig. 3.12 and 3.13). Initial fractionation with ethyl acetate (which yielded fractions 6M and 6E) enriched specific peaks (RTs = 23.2 and 24.3 = PAC dimer and trimer respectively.) This was supported by the higher intensity of signals at *m/z* 577 and 865 in their MS spectra (Fig. 3.13). There was a corresponding enrichment in certain peaks in the water fractions derived samples (F1, F3 and F4). Most notably the non PAC peaks at RT = 19.8 (*m/z* 371, caffeic acid derivative) and 22.5 min (*m/z* 495, lignan

derivative) were enriched (Karonen et al., 2004). These signals were also enriched in the MS spectra of F1 and F3 (Fig. 3.13).

The water derived fractions (F1, F3 and F4) yielded progressively more higher MW PACs as judged by the PAC-rich “hump” between 25-50 mins (Fig. 3.12). This trend is also apparent in the MS spectra (Fig. 3.13) more obviously by the relative increase in the signal at *m/z* 1153 (but also possibly *m/z* 1441) from fraction F1- F4.

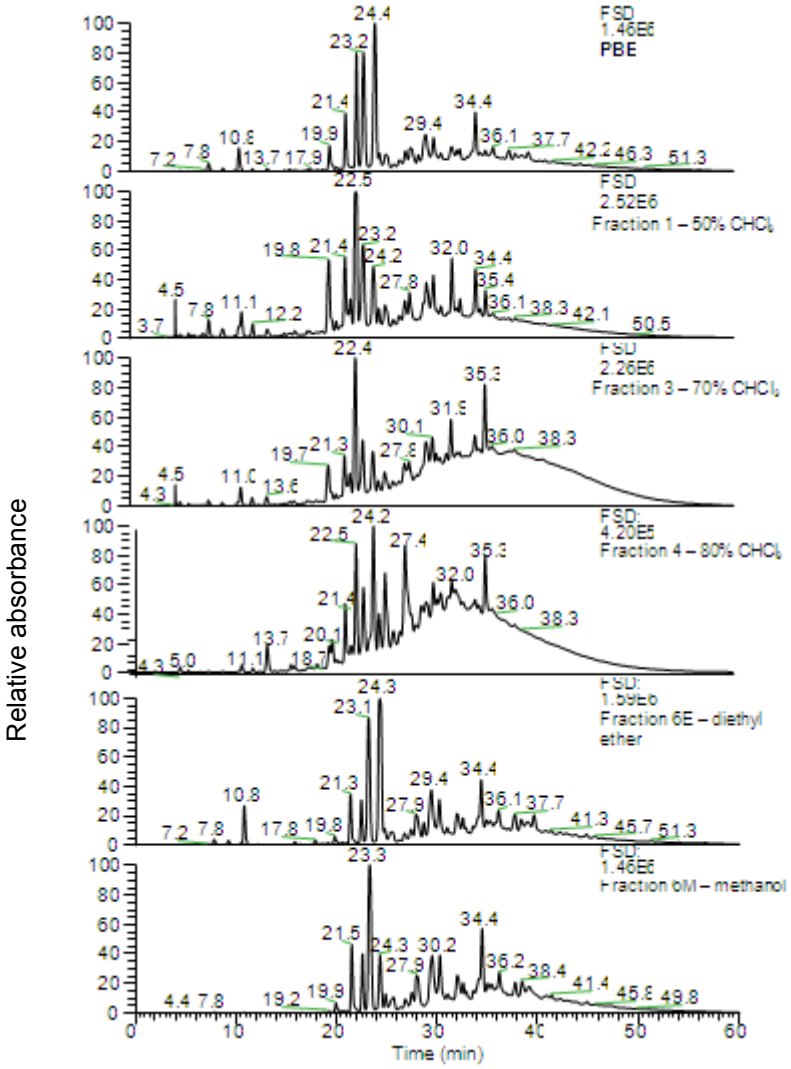


Fig 3.12: LC traces of PBE fractions obtained by solvent-to-solvent fractionation. Traces at 280 nm are shown. FSDs are shown in the top right corners of each trace.

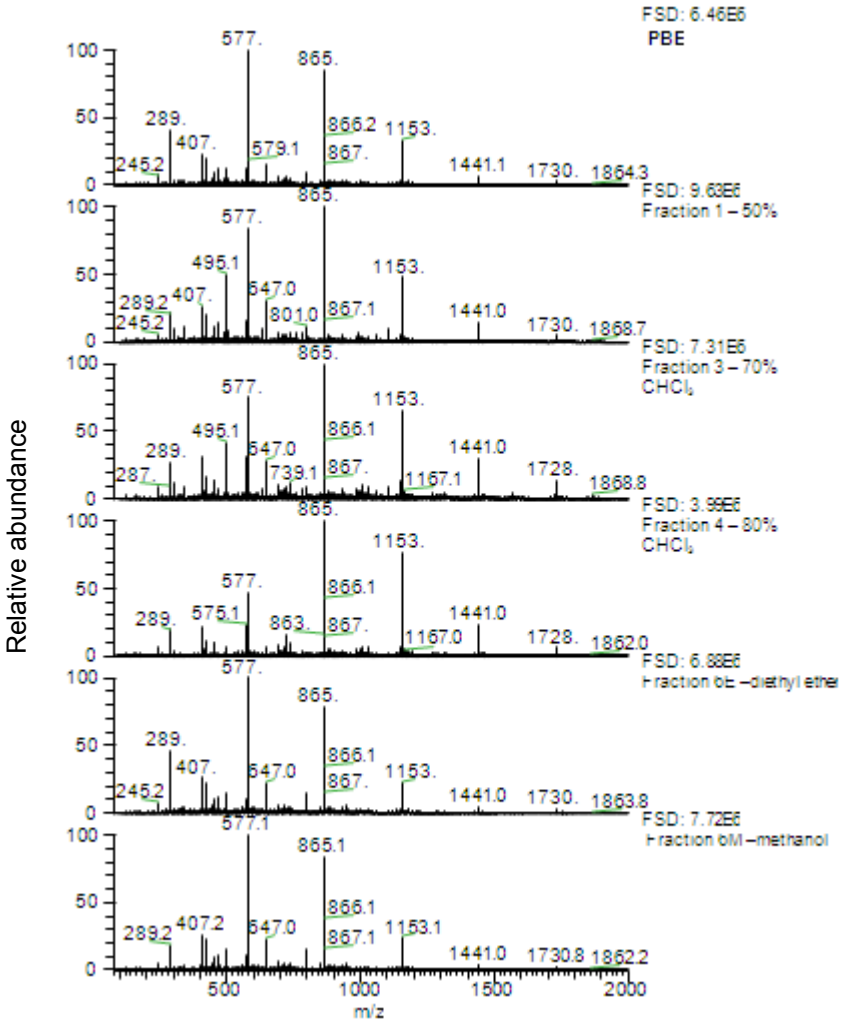
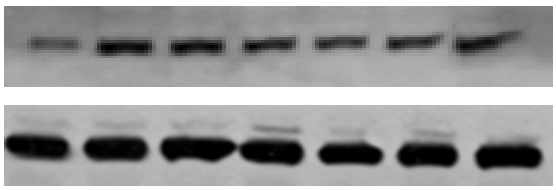


Fig 3.13: MS spectra of PBE fractions obtained by solvent-to-solvent fractionation. FSDs are shown in the top right corners of each trace.

Cell culture and western blotting suggested that all fractions tested were active with respect to phosphorylation of FOXO1a. This is shown in Fig 3.14.



−	+	−	−	−	−	−	−	+
−	−	+	−	−	−	−	−	100µg/ ml GSE
−	−	−	+	−	−	−	−	100µg/ ml GSE F1
−	−	−	−	+	−	−	−	100µg/ ml GSE F4
−	−	−	−	−	+	−	−	100µg/ ml GSE F6M
−	−	−	−	−	−	+	−	100µg/ ml PBE F4
−	−	−	−	−	−	−	+	100µg/ ml PBE F6M

Fig 3.14: Effect of GSE and PBE fractions on FOXO1a phosphorylation

Serum starved HEK293 cells were treated as above for one hour followed by lysis and SDS-PAGE as described in chapter 2. Immunoblotting was carried out using an antibody that detects FOXO1a only if it is phosphorylated on Ser 325 (pSer325 FOXO1a) and a second antibody that detects FOXO1a regardless of phosphorylation state. 'F' followed by a number denotes fraction number. 'M' denotes methanol.

3.2.6 Fractionation on Sephadex LH-20

Fractionation of the GSE and PBE samples was then carried out using a Sephadex LH20 column (as described in Chapter 2).

The fractionation of GSE on Sephadex LH20 is illustrated in Figs. 3.15 and 3.16. The unbound fractions were enriched in PAC dimers (*m/z* = 577; RT = 22.3 and 23.9) and the monomer (*m/z* = 289; RT = 25.1). Certain non-PAC derivatives were fractionated in the unbound samples including gallic acid (*m/z* = 169; RT = 10.9) and some unidentified peaks (e.g. RT = 35.1) which were concentrated during the fractionation. The MS spectra (Fig. 3.16) tend to over-report on PAC components as they ionize efficiently in negative

mode and they show the concentration in signals ascribed to monomer and dimers (*m/z* = 577 and 865 respectively). The unbound 2 sample also shows the presence of trimers (*m/z* 865).

The bound fractions were enriched in higher molecular weight PACs. This is illustrated by the relative increase in the peaks at RT = 28.3 and 30.2 (*m/z* 1153 and 865 resp) and the hump of unresolved PACs between RT 20-50 mins. The MS spectra (Fig. 3.16) also show the trend to higher molecular weight PACs with relative increases in signals at *m/z* 1153, 1441 and 1729 apparent. It was very notable that the bound samples did not contain any signals from galloylated PACs and it appears that the galloylated molecules were not recovered from the Sephadex by these treatments.

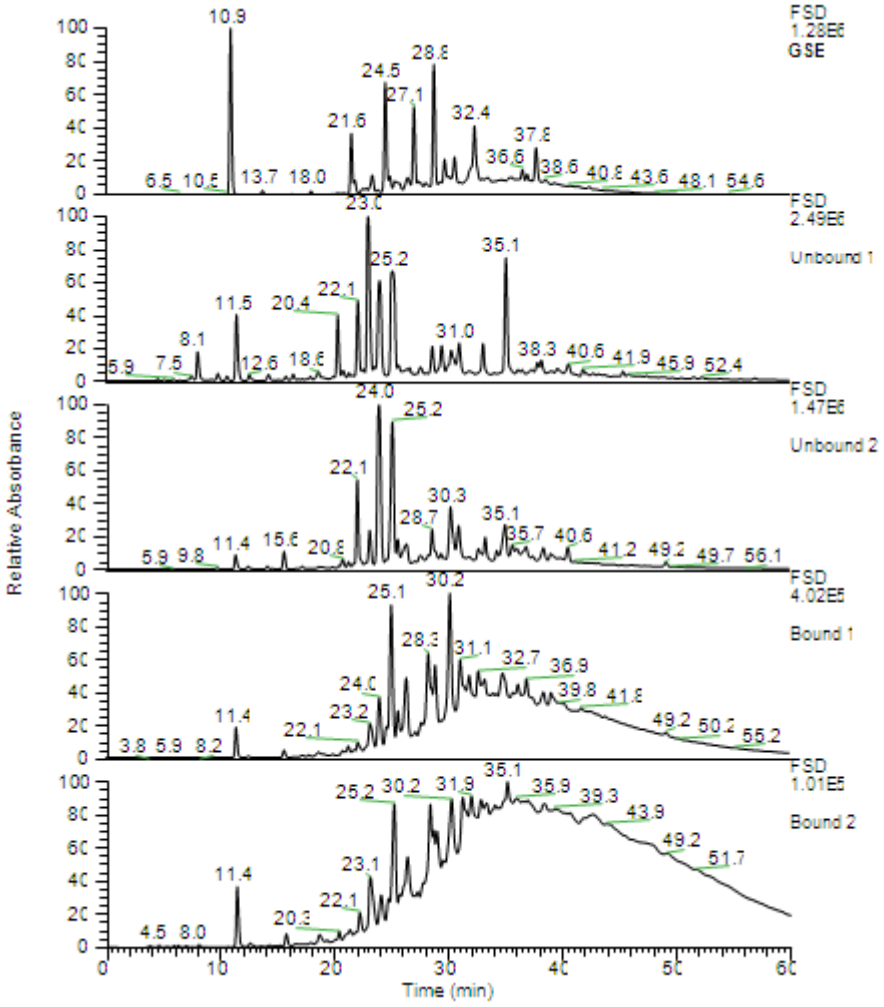


Fig 3.15: LC traces of fractions obtained by Sephadex LH20 fractionation of GSE
Traces at 280 nm are shown. FSDs are shown in the top right corners of each trace.

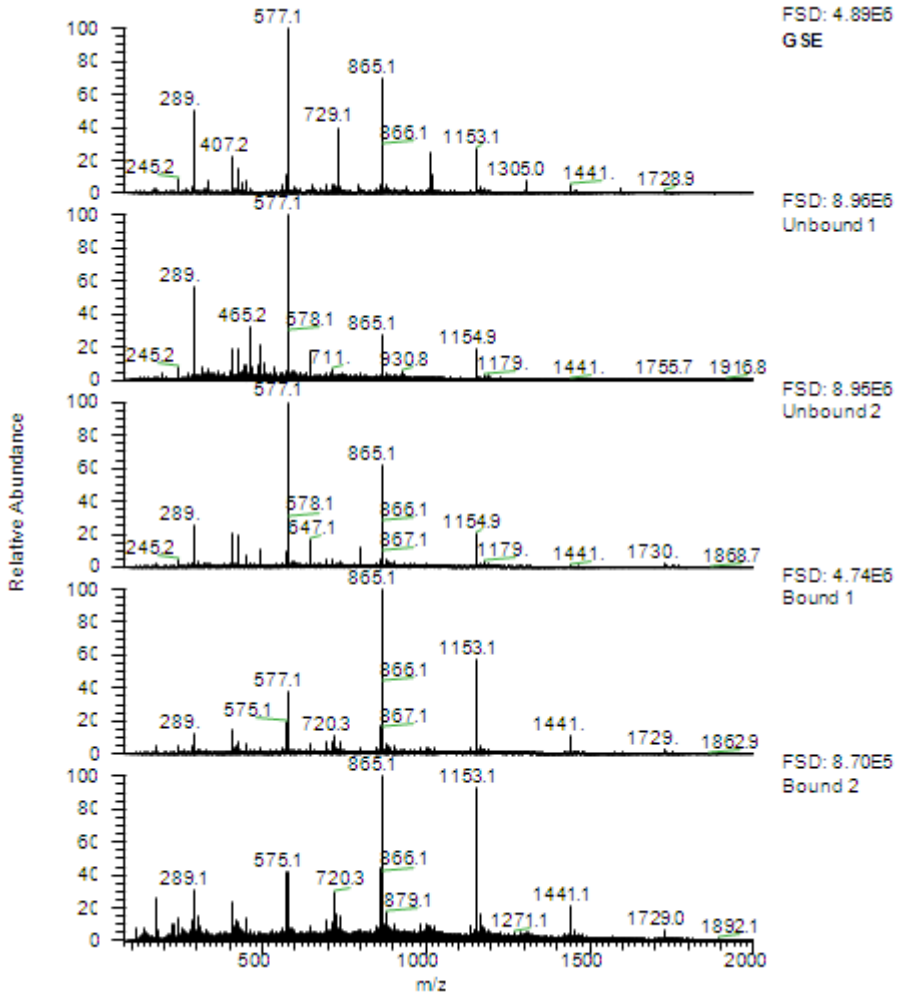


Fig 3.16 MS spectra of fraction obtained from GSE by Sephadex LH20 fractionation. FSDs are shown in the top right corners of each trace.

The fractionation of PBE on Sephadex LH20 is illustrated in Figs. 3.17 and 3.18. The LCMS run was changed to 30 min separation rather than 60 min so the traces are different from Fig. 3.9. The unbound samples were enriched in certain PACs including the PAC dimers (*m/z* 577; RT = 21.5 and 23.4) and a PAC trimer (also in RT 23.4). They also contained non-PAC components including the lignan derivatives (*m/z* 496; RT = 22.5 and *m/z* 465; RT = 28.7). Gallic acid was present (*m/z* 169; RT = 17.9) and preceding peaks (RT = 16.8 and 17.4) appear to contain the caffeic acid derivatives noted previously (Fig. 3.9). The MS spectra largely support these findings.

The bound 1 sample contained many of the peaks found in the unbound samples but also contained the characteristic hump of unresolved PAC peaks from RT 20-30 mins. The bound 2 sample was similar but appeared to contain more PACs. The MS spectra (Fig. 3.18) also illustrate the trend to higher MW PACs in the bound samples, especially in the amounts of signal at *m/z* 1153.

Fractions were prepared as aliquots of 250 µg GAE phenol content for cell culture work.

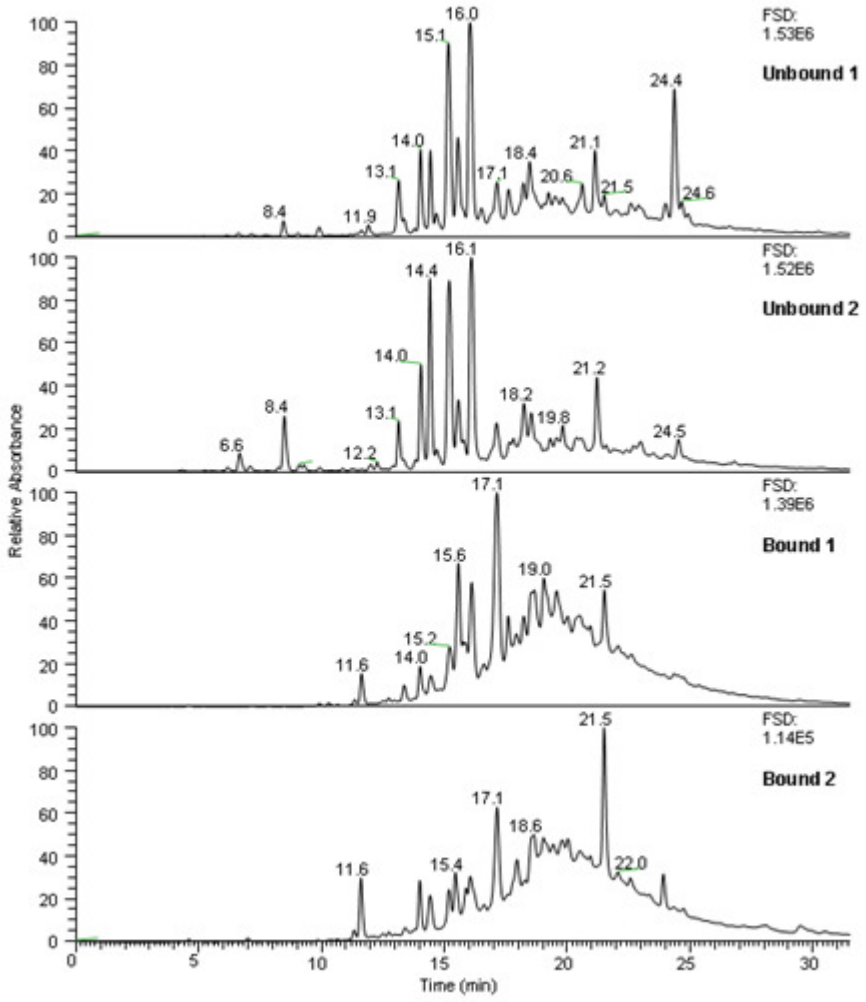


Fig 3.17: LC traces of fractions obtained from PBE by Sephadex LH20 fractionation. Traces at 280nm are shown. FSDs are shown in the top right corners of each trace.

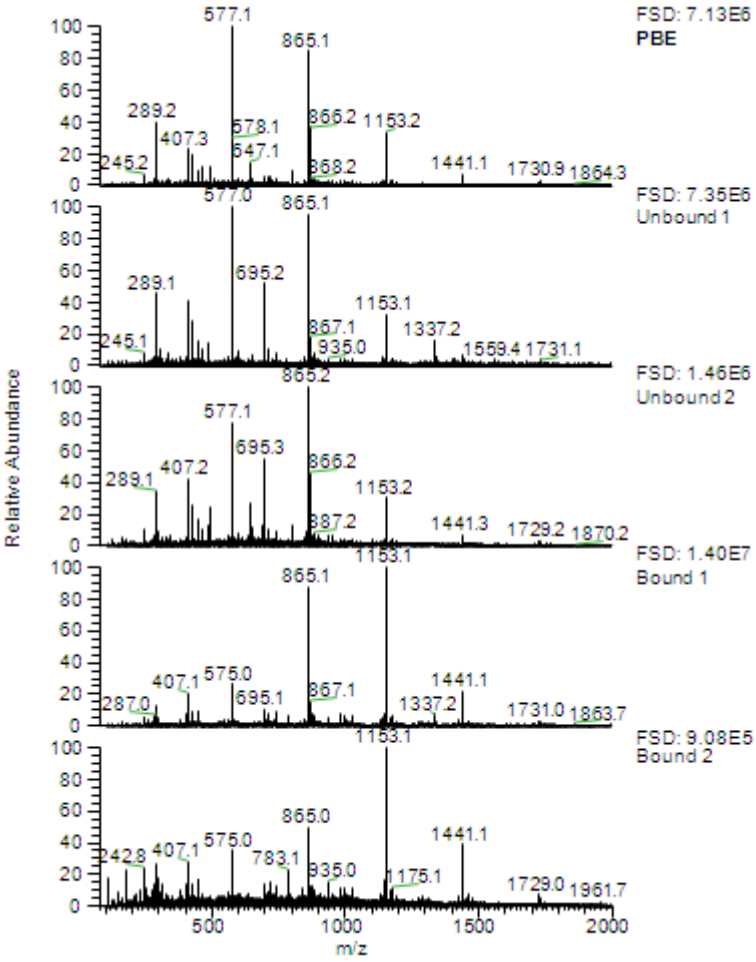


Fig 3.18: MS spectra of fractions obtained from PBE by Sephadex LH20 separation. FSDs are shown in the top right corners of each trace

The fractions from the Sephadex LH20 fractionation were applied to cells to determine the effect on FOXO1a phosphorylation. These experiments are shown in Fig 3.19 and Fig 3.20.

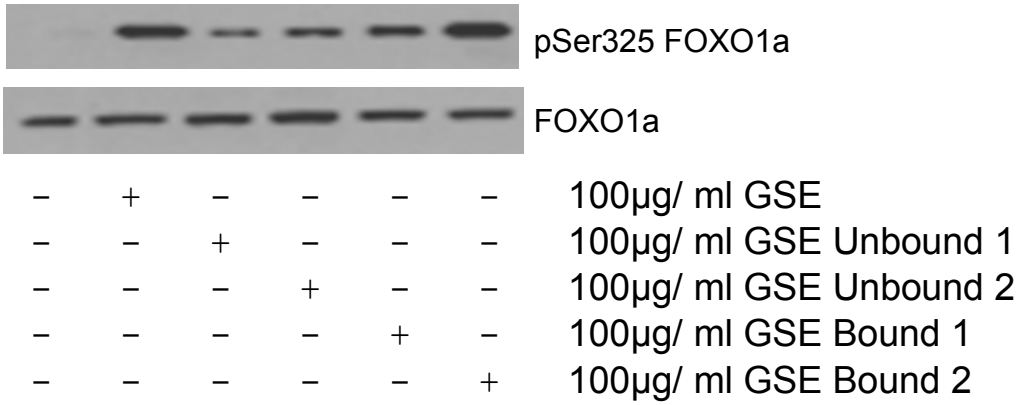
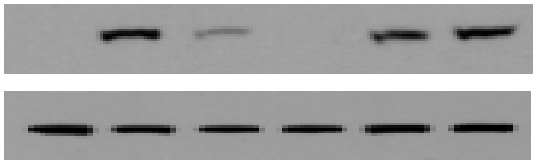


Fig 3.19: Effect of bound and unbound GSE fractions on FOXO1a phosphorylation. Serum starved HEK293 cells were treated as above for one hour followed by lysis and SDS-PAGE as described in chapter 2. Immunoblotting was carried out using an antibody that detects FOXO1a only if it is phosphorylated and Ser325 (pSer325 FOXO1a) and a second antibody that detects FOXO1a regardless of phosphorylation state (FOXO1a).

It is clear that all fractions could induce phosphorylation of FOXO1a to some extent. However, the bound fractions (and especially the bound 2 fraction) were most effective. This suggests that the higher molecular weight PACs are particularly potent as removing them (as in the unbound fractions) reduced effectiveness and retaining them recovered effectiveness.



−	+	−	−	−	−	100µg/ ml PBE
−	−	+	−	−	−	100µg/ ml PBE Unbound 1
−	−	−	+	−	−	100µg/ ml PBE Unbound 2
−	−	−	−	+	−	100µg/ ml PBE Bound 1
−	−	−	−	−	+	100µg/ ml PBE Bound 2

Fig 3.20: Effect of bound and unbound PBE fractions on FOXO1a phosphorylation. Serum starved HEK293 cells were treated as above for one hour followed by lysis and SDS-PAGE as described in chapter 2. Immunoblotting was carried out using an antibody that detects FOXO1a only if it is phosphorylated and Ser325 (pSer325 FOXO1a) and a second antibody that detects FOXO1a regardless of phosphorylation state (FOXO1a).

The pattern was similar for the PBE fractions (Fig. 3.20). In this case, the unbound fractions were less effective although the unbound 1fraction had some effect. The bound samples were the most effective and this strongly suggests that the higher MW PACs recovered in these fractions are most effective in inducing phosphorylation of FOXO1a.

3.2.7 Fractionation on polyamide columns

Another fractionation technique was then employed using a Discovery polyamide column (described in Chapter 2). Briefly, polyamide is known to adsorb highly hydroxylated molecules such as PACs under certain conditions. The unbound component of the sample was eluted from the column using 20% methanol and the bound component using 80% acetone. The fractionation of GSE by the polyamide SPE method is illustrated in Figs. 3.21 and 3.22. The unbound fractions were enriched in gallic acid (*m/z* 169; RT = 8.7) and the monomers (*m/z* 289; RT = 16.3 and 18.5). There was also

a concentration of the peak at RT = 13.4 (*m/z* 153) which may be due to dihydroxybenzoic acid. The MS spectra show the dominance of the *m/z* signals at 289 due to the enrichment of the monomers (Fig. 3.22). The signal at *m/z* 579 is also due to the presence of the monomers and results from a half-charged version of the monomers which gives a doubled *m/z* ratio.

The bound samples differed in their composition (Fig. 3.21). The bound 1 fraction was greatly enriched in the monomer peaks (RT = 16.3 and 18.4) but was relatively depleted in gallic acid (RT = 8.7) compared with the unbound samples. The bound 1 fraction also had appreciable amounts of the dimers (*m/z* 577; RT = 14.3 and 17.2). This is consistent with the first application of acetone removing the last of the unbound monomers but also enriching in some dimers. On the other hand, the bound 2 fraction was composed of a unresolved hump of PACs (RT 13-25 mins). The MS spectra (Fig. 3.22) showed these differences and confirmed that the bound 2 fraction was enriched in higher MW PACs including signals characteristic of galloylated forms which exceeded those identified in the original GSE (e.g. signals at *m/z* = 1593 and 1881 are indicative of galloylated pentamers and hexamers).

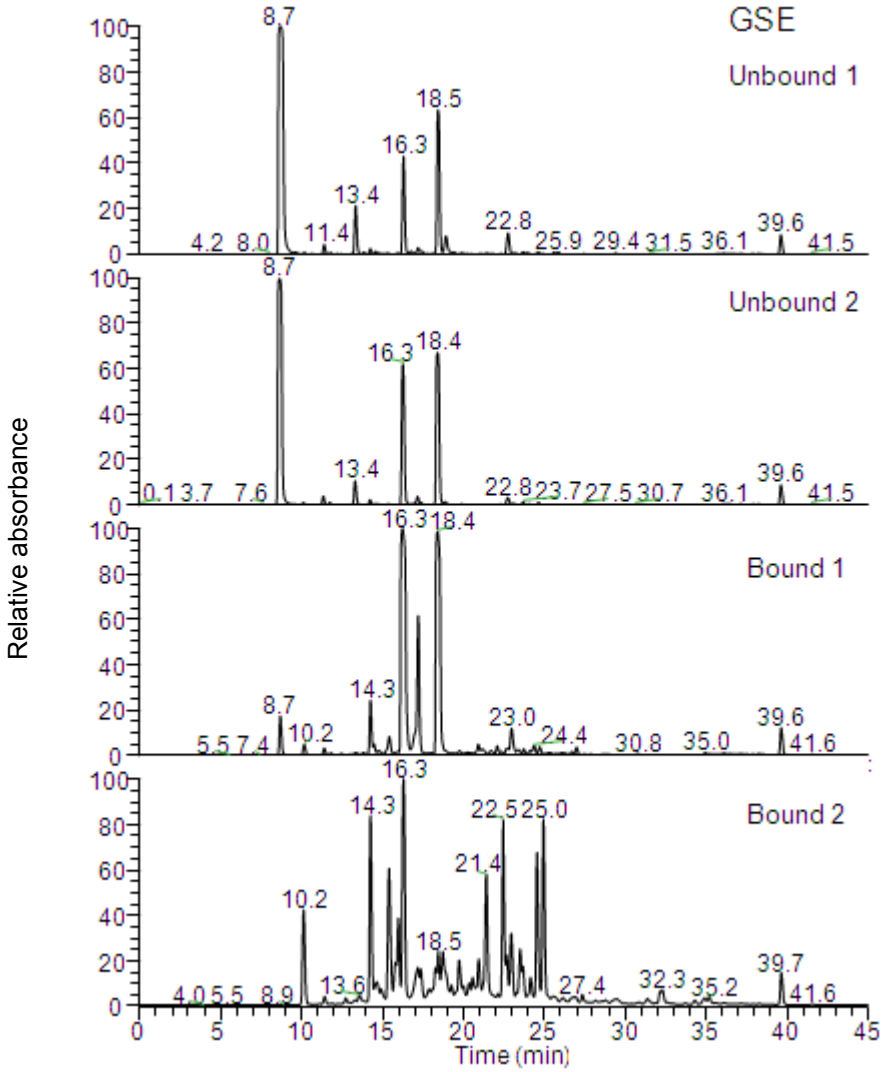


Fig 3.21: LC traces of fractions obtained from GSE from polyamide column.
Traces at 280 nm are shown. FSDs are shown in the top right corners of each trace.

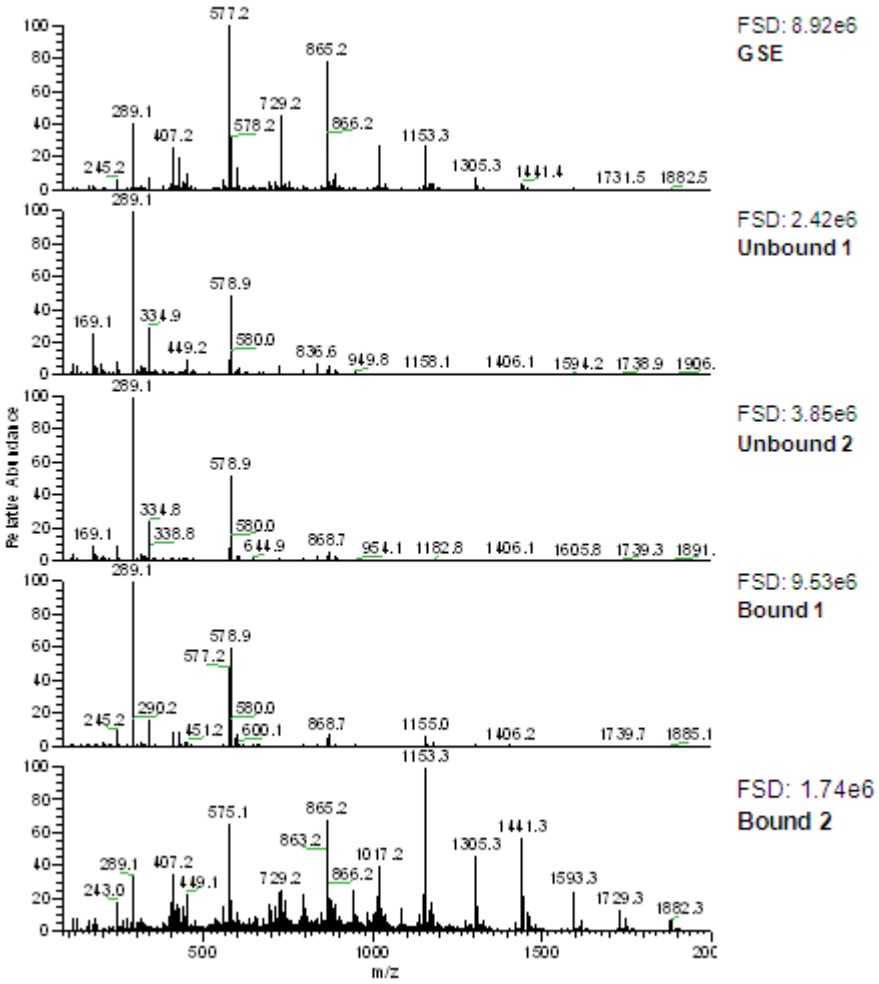


Fig 3.22: MS spectra of fractions of GSE from the polyamide column.
FSDs are shown in the top right corners of each panel.

The fractionation of polyphenol components from PBE on polyamide SPE is illustrated in Fig. 3.23 and 3.24. The original PBE sample was not run for direct comparison but the peaks can be compared with Fig. 3.9. The unbound samples were enriched in PAC components (dimers *m/z* 577; RT 14.0 and 15.1), a PAC trimer (*m/z* 865; RT = 15.5) and a monomer (*m/z* 289; RT = 16.1). The unbound fractions also contained non-PAC components including the lignan derivatives (*m/z* 495; RT = 14.0 and *m/z* 465; RT = 21.2). Gallic acid was also present (*m/z* 169; RT = 8.5). The MS spectra (Fig. 3.24) show the enrichment in dimer signals (*m/z* 577) but also pick up some trimer signals (*m/z* 865).

The bound fractions were enriched in higher molecular weight PACs. This was illustrated by the increase in the abundance of the peak at RT = 17.1 (*m/z* 1153) but also the unresolved hump of PACs between 15 and 30 mins. The MS spectra (Fig. 3.24) illustrate the trend towards higher molecular weight PACs with signals at *m/z* 1153 and 1441 becoming more dominant.

The fractions from the polyamide fractionation were applied to cells to determine the effect on FOXO1a phosphorylation. These experiments are shown in Fig 3.25.

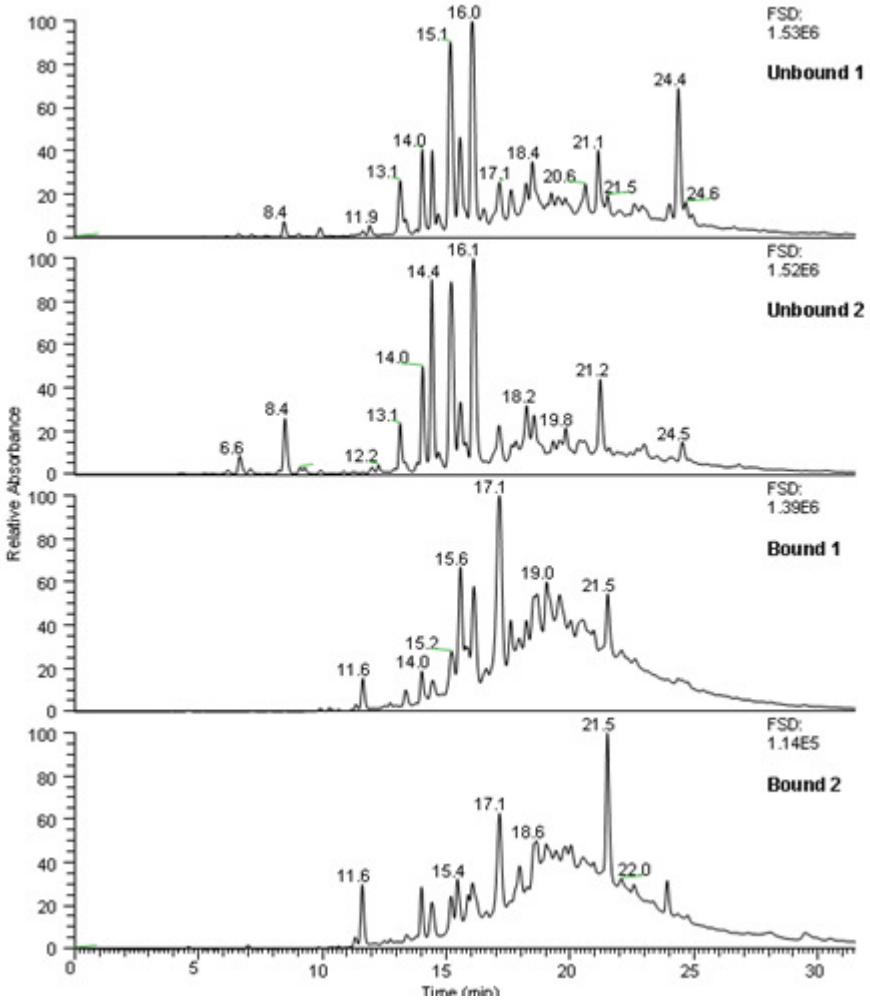


Fig 3.23: LC traces of fractions obtained from PBE on polyamide column. Traces at 280nm are shown. FSDs are shown in the top right corner of each trace.

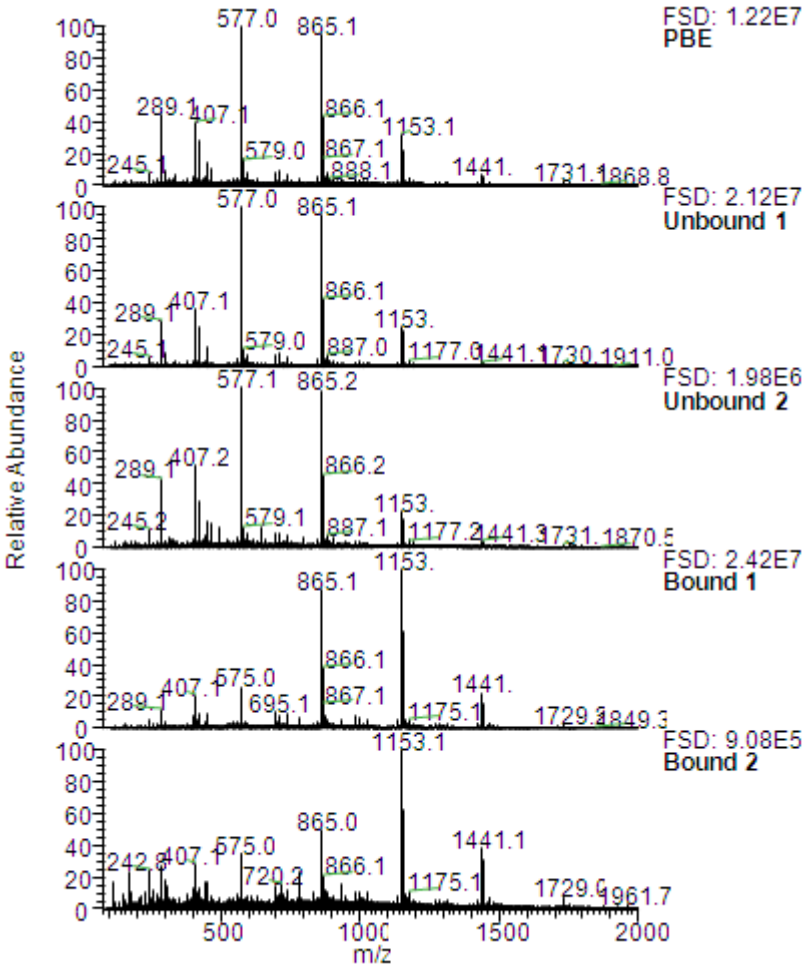


Fig 3.24: MS spectra of fractions of PBE from polyamide column.
FSDs are shown in the top right corners of each panel.

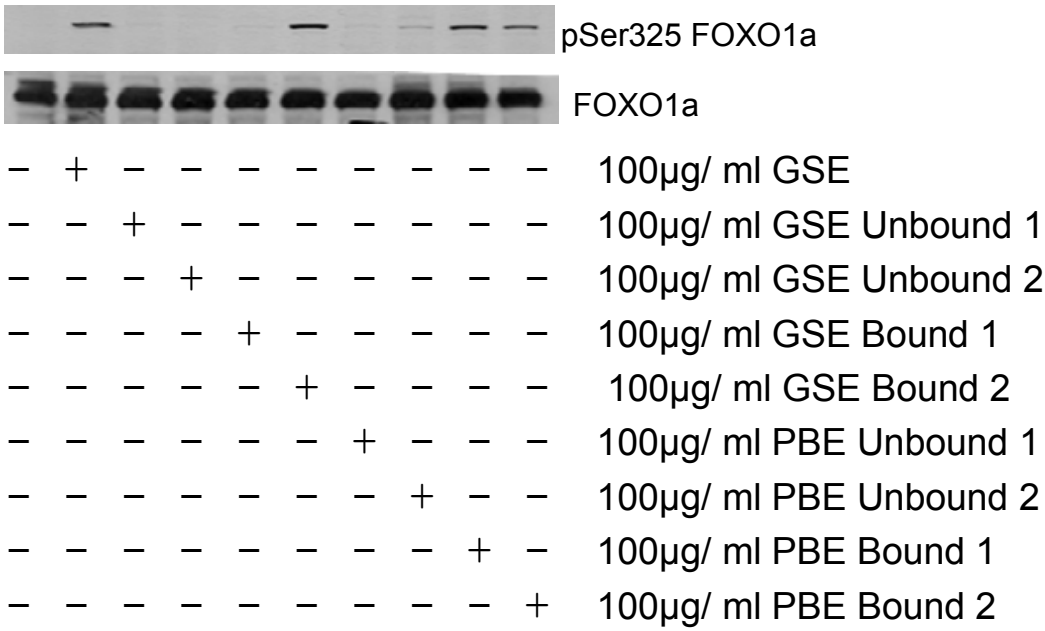


Fig 3.25: Effects on cell responses of bound and unbound fractions separated on polyamide columns.

Following fractionation on polyamide column, fractions were freeze dried, reconstituted and applied to serum starved HEK293 cells as above for one hour followed by lysis and SDS-PAGE. Immunoblotting was carried out using an antibody specific for FOXO1a only if it is phosphorylated on Ser325 (pSer325 FOXO1a) and a second antibody that detects FOXO1a regardless of phosphorylation state (FOXO1a). This again suggested the bound fractions phosphorylated FOXO1a.

Only the bound 2 fraction from the GSE polyamide fractionation was effective in inducing phosphorylation of FOXO1a (Fig 3.25). Little or no phosphorylation was observed in cells treated with the unbound 1 or 2 fractions.

The pattern was subtly different with the fractions obtained from PBE. But in essence only the bound fractions displayed appreciable ability to induce FOXO1a phosphorylation. This suggests that the higher molecular weight PACs are most effective but interestingly there seems little difference in effectiveness between the effectiveness of the non-galloylated PACs from PBE and the mixture of galloylated and non galloylated PACs from GSE.

3.2.8 Purification of PACs by normal phase HPLC

Although these fractionation methods provided evidence that PACs were important for the induction of the phosphorylation events, they do not provide particularly stringent separation of PACs as all were usually mixtures of sizes and types. Therefore, attempts to purify the PACs from GSE and PBE were made using HPLC techniques. Due to the poor quality of chromatography achieved using reverse phase HPLC, further analysis of these samples was carried out using normal phase HPLC, which has been reported to achieve baseline separation of PACs (Hellstrom, 2008). The aqueous/ organic solvent gradient of the normal phase method was altered and fine-tuned to increase separation of the peaks of interest. After some adaptations of the method, the polyamide bound fraction 1 of GSE gave some separation of peaks (Fig 3.26) but separation of peaks in the bound fraction 2 from PBE was less successful (Fig. 3.27). The peaks were tentatively identified by their MS properties (Table 3.2).

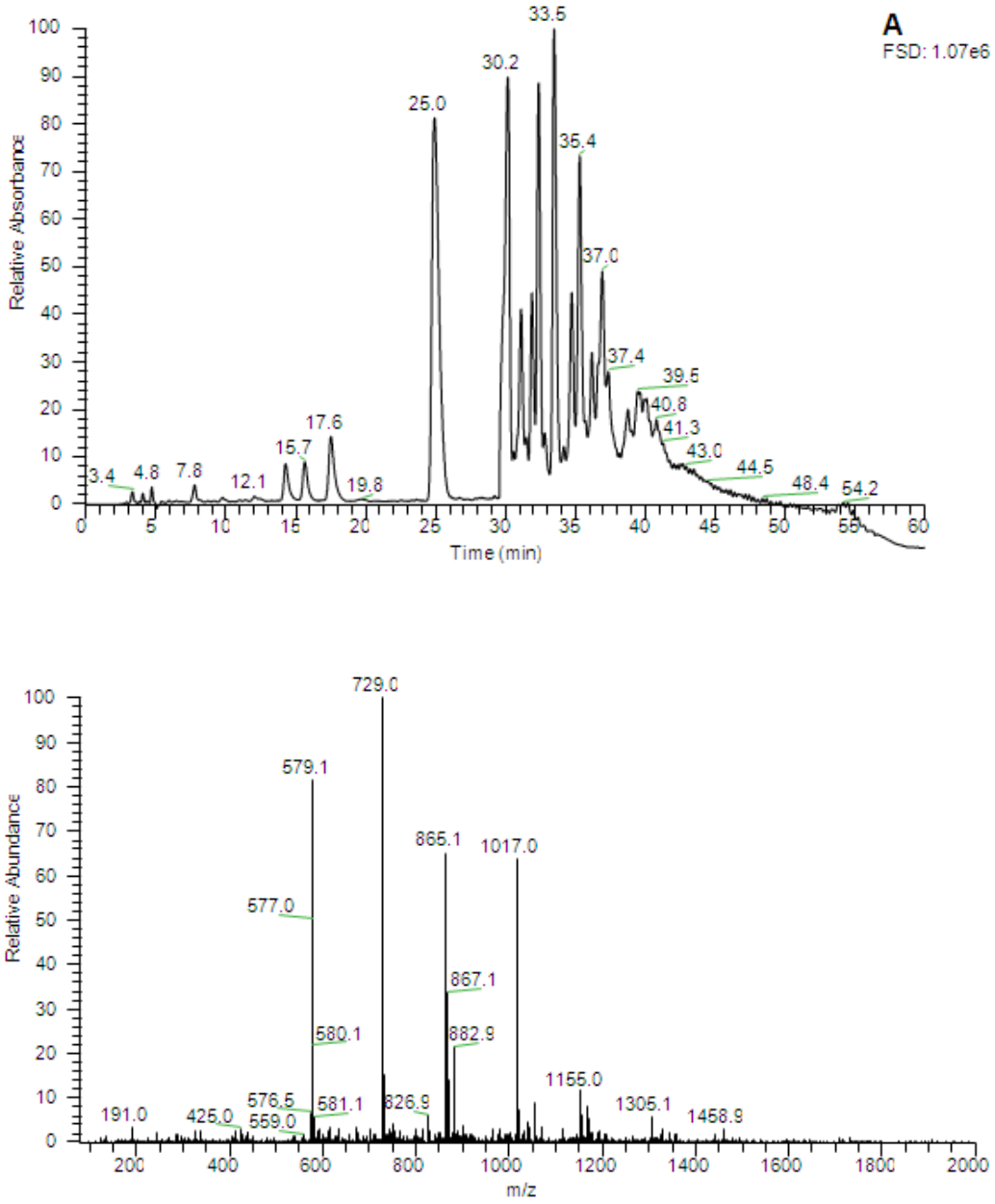


Fig 3.26: Separation of GSE by normal phase method
The sample was polyamide bound fraction 2. The trace at 280 nm is shown in panel A. The MS spectrum across the separation zone (5-60 mins) is shown in panel B. FSDs are shown in the top right corners of each panel.

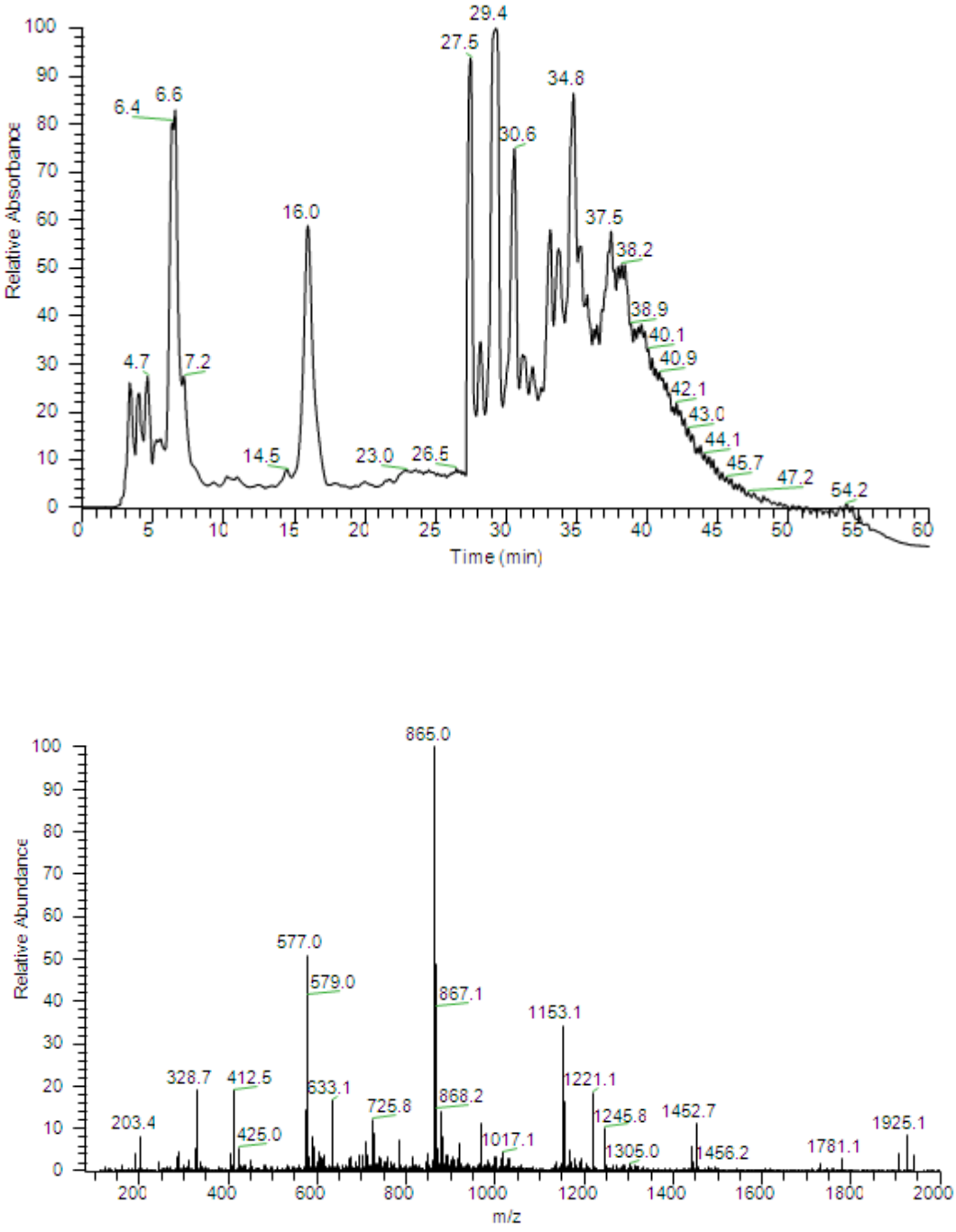


Fig 3.27: Separation of PBE by normal phase method
The sample was the polyamide bound fraction 1. The trace at 280 nm is shown in panel A. The MS spectrum across the separation zone (5-60 mins) is shown in panel B. FSDs are shown in the top right corners of each panel.

GSE bound

RT (mins)	m/ z (M- H)	Putative ID
14.2	503.0	Not identified
15.7	614.9	Not identified
17.6	578.6	dimer
25.0	882.8	A-type trimer
30.2	577.0	dimer
31.1, 31.9	579.1	dimer
32.0	577.0. 729.1	dimer, galloylated dimer
33.5	729.0	galloylated dimer
34.5	729.0, 865.0, 1055.1	galloylated gimer, trimer, not identified
35.4	729.0	galloylated dimer
35.8, 37.0, 37.4	865.1	trimer
38.5, 39.5	1017.1	galloylated trimer
40- 60	575.0, 729.0, 865.1, 1017.0 1153.1, 1151.1,1169.0, 1305.0	dimer, galloylated dimer, trimer, galloylated trimer tetramer, not identified, galloylated tetramer

PBE bound

RT (mins)	m/ z (M- H)	Putative ID
16.0	203.4	Not identified
17- 27	1908.4	Not identified
27.6	920.9	Not identified
28.2	970.2	Not identified
29.4	577.0	dimer
30.6	412.4, 579.0	Not identified, dimer
31.3	579.1, 865.1	dimer, trimer
32.0	577.0, 711.1, 729.0, 865.1, 879.1	dimer, not identified, galloylated dimer, trimer, not identified
33.2, 33.6, 34.8	865.1	trimer
36- 37	865.1, 1452.6	trimer, not identified
37.5	1153.0	tetramer
38.2	328.7	Not identified
39- 60	577.0, 633.1, 863.1, 1153.1, 1441.0	dimer, not identified, trimer, tetramer, pentamer

Table 3.2: Putative identification of compounds in bound fractions prepared on the new normal phase gradient

Chromatograms and MS data are shown in Figs 3.26 and 3.27 respectively.

However, the ionisation in the mixture of solvents used for the normal phase method was not very reliable and the MS data was not convincing. Therefore, individual peaks were collected as they eluted from the UV detector, dried to remove the solvents and re-analysed using direct-infusion into the MS detector (Figs. 3. 28 and 3.29). Their putative identities are noted in Table 3.3.

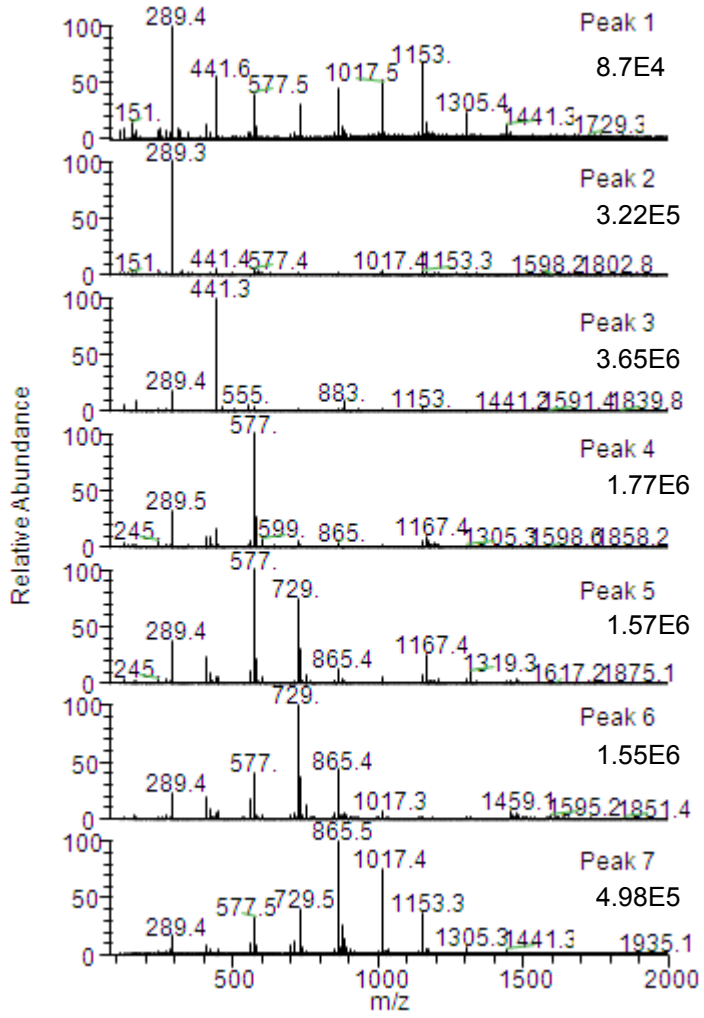


Fig 3.28: MS spectra of peaks purified from GSE using normal phase HPLC

Peaks were dried, resuspended then subjected to ESI-MS. The FSDs are given in each panel

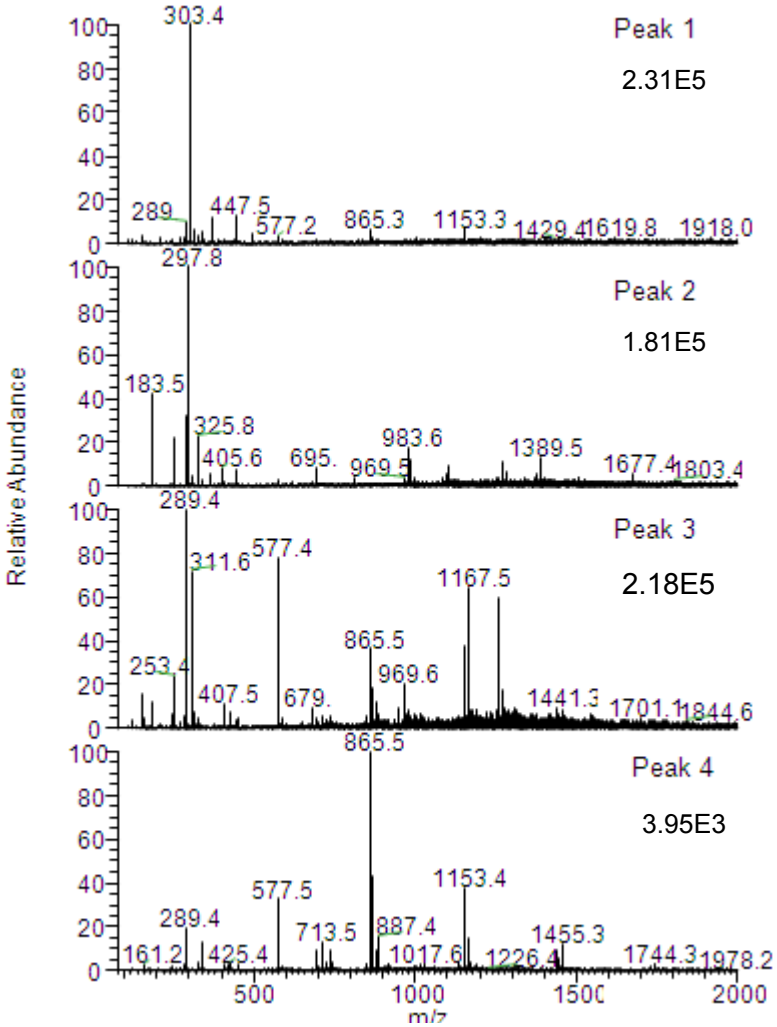


Fig 3.29: MS spectra of purified PBE peaks.

FSDs are shown in the top right corners of each panel.

GSE bound

Peak no	RT (mins)	m/ z (M- H)	Putative ID
1	25.0	289.4, 441.6, 577.5, 729.1, 865.1, 1017.5, 1153.1, 1305.4, 1441.3, 1729.0	monomer, galloylated monomer, dimer, galloylated dimer, trimer, galloylated trimer, tetramer, galloylated tetramer, pentamer, galloylated pentamer
2	30.3	289.3	monomer
3	32.4	441.3	galloylated monomer
4	33.5	577.4	dimer
5	35.4	577.4, 729.4	dimer, galloylated dimer
6	37.0	577.4, 729.4, 865.4	dimer, galloylated dimer, trimer
7	40.8	289.4, 577.5, 729.5, 865.5, 1017.4, 1153.3	monomer, dimer, galloylated dimer, trimer galloylated trimer, tetramer

PBE bound

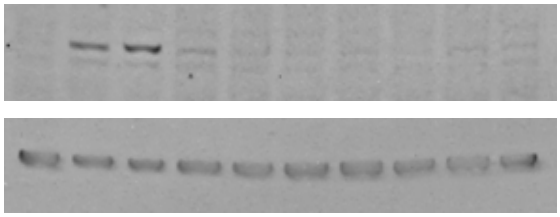
Peak no	RT (mins)	m/ z (M- H)	Putative ID
1	22.75	303.4	Not identified
2	29.4	297.8	Not identified
3	30.7	289.4. 311.6, 577.4, 865.4, 969.6, 1167.5, 1267.4	monomer, not identified, dimer, trimer, not identified, not identified, not identified
4	34.8	289.4, 577.5, 865.5, 1153.4, 1455.3	monomer, dimer, trimer, tetramer Not identified

Table 3.3: Putative identification of compounds in purified peaks prepared on the new normal phase gradient

MS data is in Figs 3.28 and 3.29 for GSE and PBE respectively. The compounds found in each fraction were identified from Passos, 2007.

In some cases, e.g. GSE peaks 2, 3 and 4, the separation appears to yield relatively pure components whereas other peaks contain mixtures of higher MW PAC components. The PBE separation was less effective.

Cell culture and western blotting suggested none of these purified GSE samples strongly induced the phosphorylation of FOXO1a (Fig 3.30). Similar results were obtained with PBE (data not shown).



−	+	−	−	−	−	−	−	−	−	Positive control
−	−	+	−	−	−	−	−	−	−	100µg/ ml GSE Bound 2
−	−	−	+	−	−	−	−	−	−	100µg/ ml GSE Bound 2 P1
−	−	−	−	+	−	−	−	−	−	100µg/ ml GSE Bound 2 P2
−	−	−	−	−	+	−	−	−	−	100µg/ ml GSE Bound 2 P3
−	−	−	−	−	−	+	−	−	−	100µg/ ml GSE Bound 2 P4
−	−	−	−	−	−	−	+	−	−	100µg/ ml GSE Bound 2 P5
−	−	−	−	−	−	−	−	+	−	100µg/ ml GSE Bound 2 P6
−	−	−	−	−	−	−	−	−	+	100µg/ ml GSE Bound 2 P7

Fig 3.30: Effect of peaks purified from GSEon phosphorylation of FOXO1a.
Samples from each peak were collected directly from the mass spectrometer, dried and reconstituted. Serum starved HEK293 cells were treated as above followed by lysis and SDS-PAGE. Immunoblottting was carried out using an antibody that detects FOXO1a only if it is phosphorylated on Ser325 (pSer325 FOXO1a) and a second one detects FOXO1a regardless of phosphorylation state.

However, when these purified samples were re-analysed by LCMS, it was found they had deteriorated significantly (Fig. 3.31).

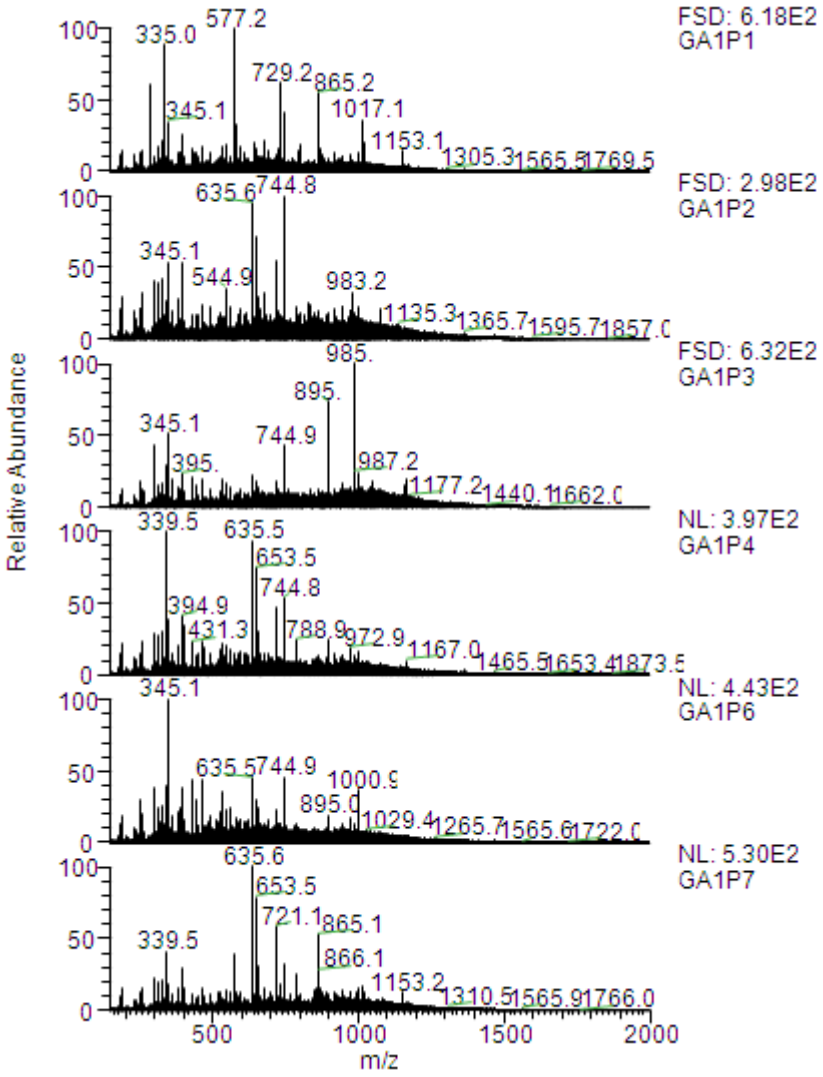
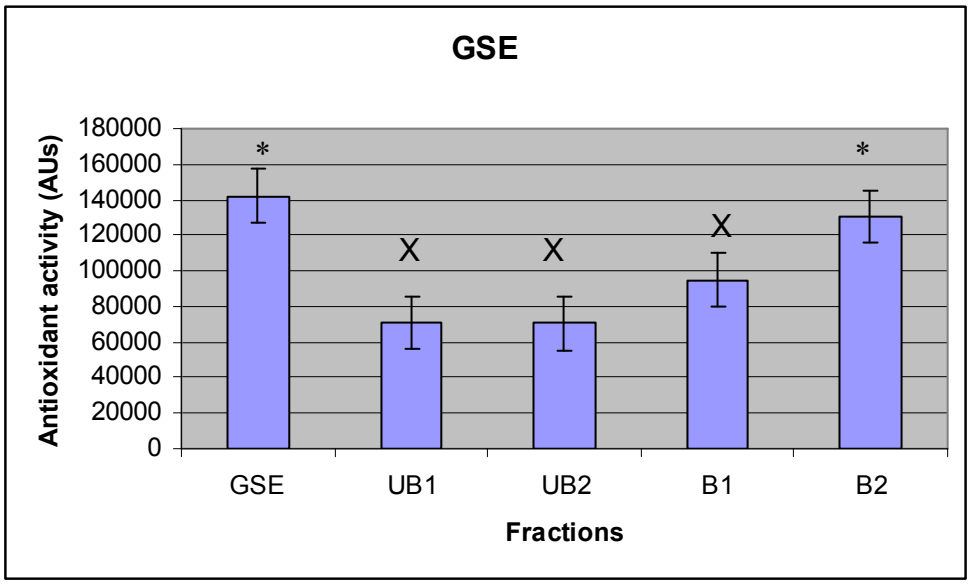


Fig 3.31: Purified samples after one week in storage. Purified samples appear to be unstable compared to unpurified samples.

No P5 sample was available for this analysis. Few of the original MS signals were present and the signal MS intensity has been greatly reduced. This may be because the PAC components are less stable when isolated than when in a complex mixture.

3.2.9 Antioxidant activity of PAC fractions

FRAP assays (as described in Chapter 2) were carried out to assess the antioxidant capacity of the various fractions harvested from the polyamide columns. Although the active samples that induced phosphorylation of FOXO1a and AMPK had considerable antioxidant capacity so did many of the fractions that were not active. This is illustrated graphically in Fig 3.32. The samples found to be active are marked with an asterisk and those found to be inactive with a cross. Active and inactive fractions both have considerable antioxidant capacity, suggesting that if antioxidant capacity does play a part in FOXO1a and AMPK phosphorylation, it is unlikely to be the only mechanism involved.



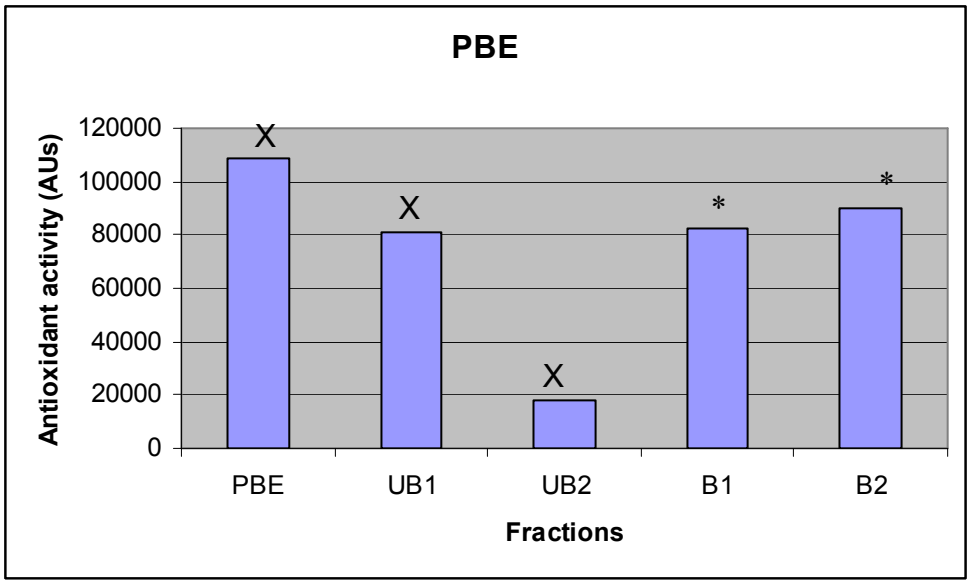


Fig 3.32: Antioxidant capacity of GSE and PBE fractions.

* = active fractions; X = inactive fractions

This theory is supported by the fact that GSE, which was more effective than PBE in inducing FOXO1a phosphorylation, has similar FRAP levels to PBE.

3.2.10 Metals enhance effects of proanthocyanidins on AMPK and FOXO1a phosphorylation

Previous results found that insulin-like properties of tea polyphenols may be potentiated by zinc (Cameron, 2010). Therefore the effect of GSE was reanalysed in the presence and absence of zinc (Fig 3.33).

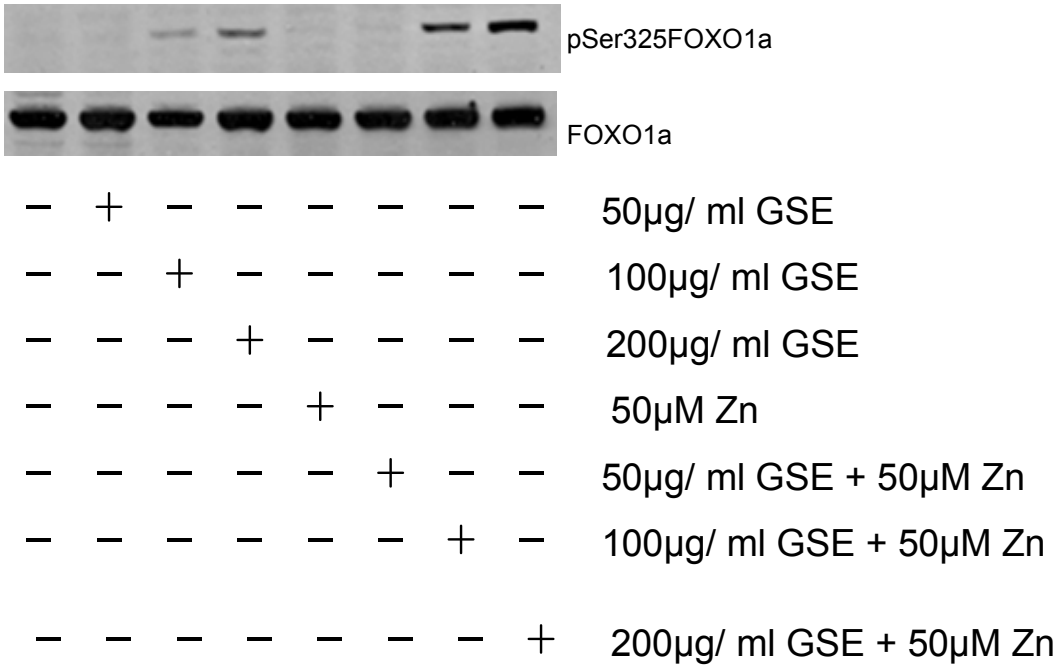


Fig 3.33: Western blot showing effects of zinc on the activity of GSE

Serum starved HEK293 cells were treated as above with GSE in the presence and absence of zinc followed by lysis and SDS- PAGE. Immunoblotting was carried out using an antibody that detects FOXO1a only if it is phosphorylated on Ser325 (pSer325 FOXO1a) and a second one that detects FOXO1a regardless of phosphorylation state (FOXO1a).

These results suggest that the presence of zinc enhanced the GSE induced phosphorylation of FOXO1a.

3.3 Discussion

3.3.1 What structural components are important for the ability to induce phosphorylation of FOXO1a

GSE was more effective than PBE in inducing phosphorylation of FOXO1a and AMPK. The initial LCMS analysis showed that GSE contained a higher proportion of proanthocyanidins (PACs) than PBE, which also contained non-PAC components such as lignans and caffeic acid derivatives. This suggested that the PACs were important for effectiveness. Indeed, the most effective fractions after fractionation using Sephadex LH20 and polyamide SPE were those enriched in PACs, which strongly suggests that these were active agents.

The higher proportion of GA and galloylated molecules in GSE than in PBE could be a contributory factor to the greater effectiveness of GSE. However, it is unlikely to be the only factor that controls the induction of FOXO1a and AMPK phosphorylation as galloylated molecules were found in some inactive fractions and some of the active fractions contained only a low concentration of GA and galloylated molecules. For example, fractionation of GSE on Sephadex LH20 produced a bound fraction that lacked galloylated PACs (Fig. 3.15 and 3.16), probably because the galloylated PACs had remained attached to the Sephadex. This fraction was effective in inducing phosphorylation of FOXO1a at 100 μg/ml. The GSE bound 2 fraction obtained after polyamide SPE was particularly enriched in galloylated PACs and contained high MW forms that were not apparent in the original GSE. This fraction was also effective in inducing phosphorylation of FOXO1a at 100 μg/ml. The solvent to solvent fractionation also produced fractions that

were relatively enriched in galloylated PACs but these were no more effective than other fractions at the levels tested. However, in this case, the fractions always contained a wide mix of components and lacked stringency. If time had allowed, a more detailed comparison of the different PAC-rich fractions at a range of concentrations may have suggested more detailed structural requirements for activity. However, the approach taken was to separate and purify PACs and then directly compare their effectiveness. Unfortunately, although the normal phase method did achieve some separation, this approach took much longer than expected to successfully separate the PACs. The instability of the purified PACs was unexpected. Oxidative degradation of polyphenols is well known but seemed to be accelerated possibly because they were both pure and at low concentration.

It was also noted that galloylated molecules eluted from the C18 HPLC columns close to the end of the run and are probably more hydrophobic than their ungalloylated forms. Hydrophobicity may increase their uptake through the plasma membrane and make them more effective in inducing intracellular insulin-like phosphorylation events than their ungalloylated counterparts. Indeed, some galloylated tea components such as EGCG are predicted (e.g. by *Molinspiration* software) to be more hydrophobic than their ungalloylated forms (EGC). Whilst this thesis was in preparation, another group reported that GSE PACs induced IR autophosphorylation. The downstream phosphorylation pattern was different compared with insulin, with more sustained MAPK activation than insulin is capable of (Montagut, 2010). More research is needed to confirm therefore how the intracellular effects

observed are mediated and whether or not more than one agent in the extract is involved. For example, it would be interesting to track if these PACs enter the cell or act at the cell surface perhaps through interactions with membrane-sited receptors or the plasma membrane itself (Fraga *et al*., 2010).

Because the affinity of tannins for proteins and potentially for receptors has been proposed to increase with an increasing degree of polymerisation (DP) *in vitro* due to a corresponding increase in ability to interact due to hydrogen bonding or crosslinking (Bate-Smith, 1973), fractionation based on size, using the method developed by Saucier *et al*, 2001, was attempted. This led to the conclusion that degree of polymerisation was not a major factor as fractions containing PACs of all sizes were equally able to induce the phosphorylation of FOXO1a at 100 μg/ml phenol content, It should be noted that the LCMS methods used can only detect molecules of $m/z < 2000$ and some of these fractions may contain PACs of much greater molecular weight (Hellstrom *et al*, 2008).

3.3.2 Antioxidant activity may be contributory factor to insulin-like effects

Although the active bound samples possessed significant antioxidant activity so did many of the inactive ones. Therefore it seems that antioxidant activity is unlikely to be the sole or major mechanism by which these compounds induce phosphorylation of FOXO1a and AMPK but that it may be important but insufficient for the effect. A future approach would be to rank the antioxidant potential of PAC-rich samples with established varying levels of effectiveness.

3.3.3 Zinc enhances the activity of these compounds by an unknown mechanism.

The presence of zinc was found to enhance the FOXO1a phosphorylating effects of these samples but a mechanism of action was not determined. Previous work in this area has suggested the site of action to be at or upstream of PI3K (Cameron *et al,* 2008) and that a tropolone like structure was necessary for this effect (Cameron *et al,* 2010).

This study found for salicylate on its own to phosphorylate ACC and AMPK and to dephosphorylate S6 a free 2-OH group was required. Further work investigating this and other structures with a tropolone or tropolone like motif may therefore be of potential benefit.

This study also found antioxidant activity may play a part in some of the effects observed therefore it is also possible that the presence of a divalent cation my facilitate reducing and therefore antioxidant potential. Further screening in this area in the future may also be potentially beneficial.

CHAPTER 4 – BENZOIC ACIDS

4.1 Introduction

4.1.1. Antihyperglycaemic properties of hydroxybenzoic acids

The difficulties that were encountered in chapter 3 in purifying individual compounds to homogeneity encouraged an alternative approach to the study of compounds regulating the signalling pathways of interest. This chapter took a candidate-based approach, starting with the observation that gallic acid (GA, 3,4,5-trihydroxybenzoic acid), a component of both extracts studied in the previous chapter, induced effects similar to those of metformin on AMPK, ACC and S6 phosphorylation status.

Amongst the other hydroxybenzoic acids, salicylic acid (SAL) and its acetylated form, aspirin, have been known for many years to exhibit anti-hyperglycaemic effects at high concentrations (Williamson, 1901; Yuan *et al*, 2001). The mechanism of action is unclear. One study linked the anti-hyperglycaemic properties of salicylate to suppression of hepatic gluconeogenesis, suggested to be a result of mitochondrial inhibition (Woods *et al*, 1974) as SAL is a known inhibitor of mitochondrial energy production (Brody and Fouts, 1956). SAL inhibits the mitochondria in a similar manner to the uncoupling agent 2,4-dinitrophenol (DNP), by abolishing ATP production but not oxygen consumption. However the two agents differ in that millimolar concentrations of SAL are required to generate effects similar to micromolar amounts of DNP (Brody and Fouts, 1956). In the last twelve years however, inflammatory signalling mechanisms, particularly SAL-dependent inhibition of TNF-alpha-induced NFkB signalling have become an increasingly prominent explanation for anti-hyperglycaemic properties of SAL (Yin *et al,* 1998). Other

workers have found that TNF-alpha itself suppresses gluconeogenesis, indicating that it will be hard to reconcile anti-inflammatory mechanisms with those that depend on inhibition of gluconeogenesis (Grempler *et al*, 2004). Comparative investigation of a panel of hydroxybenzoic acids offers the possibility of differentiating between these two mechanisms as, for example, the related 2, 5-dihydroxybenzoic acid (as sodium gentisate) exhibits anti-inflammatory properties (Meyer and Ragan, 1948) similar to SAL but does not exhibit anti-hyperglycaemic properties (Smith and Meade, 1952). The mechanism or target(s) underlying these differences is unclear at present. To investigate this further, commercially-available pure samples of gallic acid (GA) and related analogues were investigated for effects on AMPK, ACC and S6 phosphorylation status. A panel of other benzoic acids and related analogues which have been shown to be metabolites produced following the ingestion of proanthocyanidin-rich extracts and their subsequent breakdown by gut microflora in rats (Gonthier *et al*, 2003) and in humans (Appeldoorn *et al*, 2009),was also tested.

4.1.2 Benzoic acids

Benzoic acids (BA) consist of a benzene ring with an acid group as described in Chapter 3. Some hydroxylated BAs are shown in Fig 4.1:

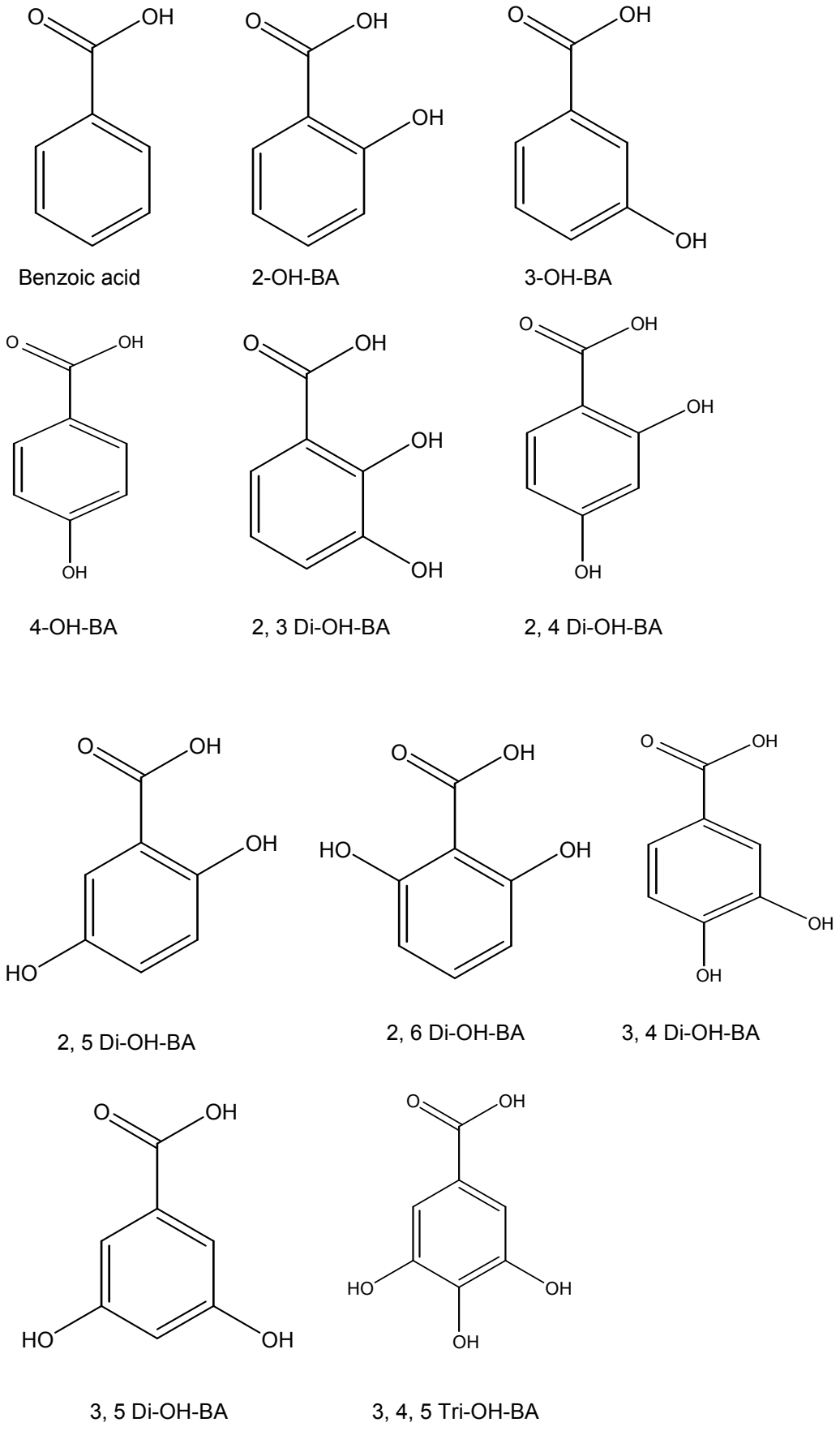


Fig 4.1: Some hydroxybenzoic acids studied in this chapter

4.1.3 Gallic acid

In addition to the samples mentioned in Chapter 3, gallic acid is also found in: some oak species (Mammela *et al*, 2000); *Caesalpinia mimosoides,* a plant native to Thailand where it is used as a vegetable (Chanwitheesuk *et al*, 2007); *Drosera capesis* or Sundew, a carnivorous plant (Kovacik *et al*, 2012) sometimes used to treat respiratory disease (Hirsikopi *et al*, 2002); *Rhodeala rosea* or golden root (Ming *et al*, 2005) which has been proposed to increase lifespan in *D. melanogaster* (Jafari *et al*, 2007); *Toona sinensis* (Chinese mahogany) which has been proposed as an anti-cancer agent with gallic acid suggested to be the active component (Chen *et al*, 2009); *Arctostaphylos*. *uvi- ursi (bear*berry) which has been reported to be beneficial in diseases of the urinary tract (Pegg *et al*, 2008); Blackberry (Hager *et al*, 2002); *Threobroma cocao (*cocoa) (Osman *et al*, 2004) whose seeds are used to make chocolate; *Juglans regia* (walnut) (Muir *et al*, 2011). Mango (Elzaawely and Tawata, 2010) which has been proposed to have antidiabetic properties (Gupta and Gupta, 2011); *Emblica officianalis* (Indian gooseberry) (Sawant *et al*, 2010) which has been reported to have effects that could be beneficial in a variety of human diseases (Khan, 2009) and *Syzgium aromaticum* (clove) (Cai and Wu, 1996).

4.1.4 Fate in the body

A study in rats by Booth *et al*, (1959) suggested that gallic acid underwent methylation *in vivo* as the major urinary metabolite produced following a diet containing 0.5% gallic acid was 4-O-methyl benzoic acid. It was proposed that the source of methyl groups was from choline and a later study

suggested a retarded growth rate observed in young chicks reared on a diet relatively high in tannins could be alleviated by supplementing the diet with choline and methionine (Potter and Fuller, 1968). It was also noted by Booth (1959) that when rats were given 100 mg gallic acid by intraperitoneal injection, an additional metabolite, pyrogallol, was produced presumably due to decarboxylation of gallic acid.

4.2 Results

4.2.1 Investigation of effects of HBAs on ACC, AMPK and S6 phosphorylation.

Gallic acid was tested for its effects on the induction of phosphorylation of FOXO1a and AMPK. To test this H4IIE cells were treated with 10 mM GA and a variety of related benzoic acids. The results of one such experiment are shown in Fig 4.2.

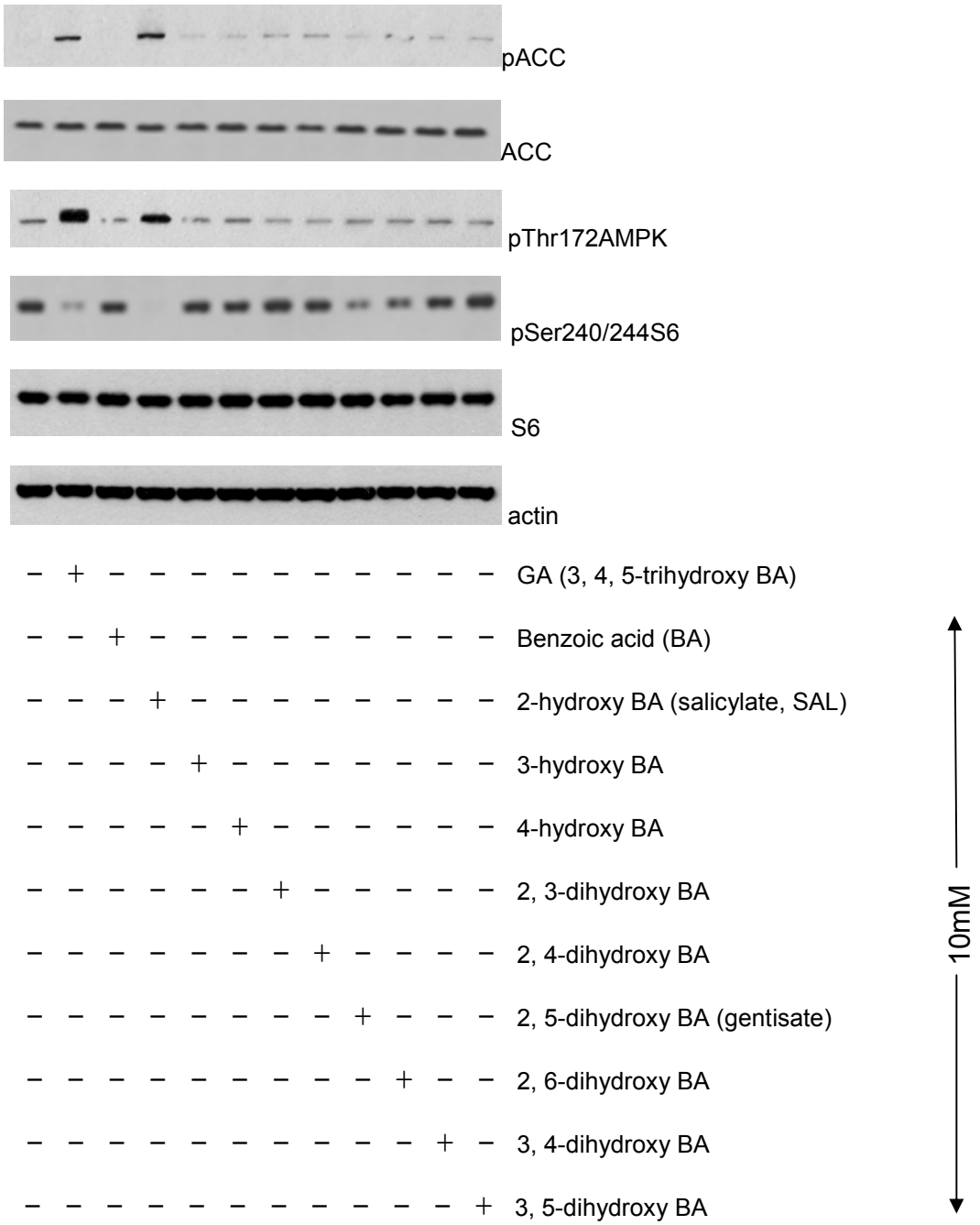


Fig 4.2: Effect of HBAs on AMPK, ACC and S6 phosphorylation

H4IIE cells were serum starved for 2 hours then treated with the HBAs shown above for 3hrs followed by lysis and SDS- PAGE. Immunoblotting was carried out using 2 ACC antibodies- one which detects total ACC (ACC) and one which detects phosphorylated ACC (pACC), an antibody which detects AMPK only if it is phosphorylated on Thr172 (pThr172 AMPK), 2 S6 antibodies- one which detects total S6 (S6) and one which detects Ser240/244 S6 phosphorylation (pSer240/244 S6) and an actin antibody.

This result indicated that only GA and SAL induced phosphorylation of AMPK. GA and SAL also reduced S6 phosphorylation. Partial effects on S6 were also observed with other HBAs, 2,5 di-OH-BA and 2,6-di-OH-BA.

4.2.2 Dose response of GA on AMPK, ACC and S6 phosphorylation

The two most active HBAs, GA and SAL, were characterised in more detail. To test the ability of GA to affect AMPK, ACC and S6 phosphorylation, a dose response experiment was carried out. This experiment found that GA induced phosphorylation of AMPK and ACC at concentrations above 1 mM. A similar dose response was observed for inhibition of S6 phosphorylation in that phosphorylation of ACC and AMPK and dephosphorylation of S6 increased with GA concentration applied (Fig. 4.3).

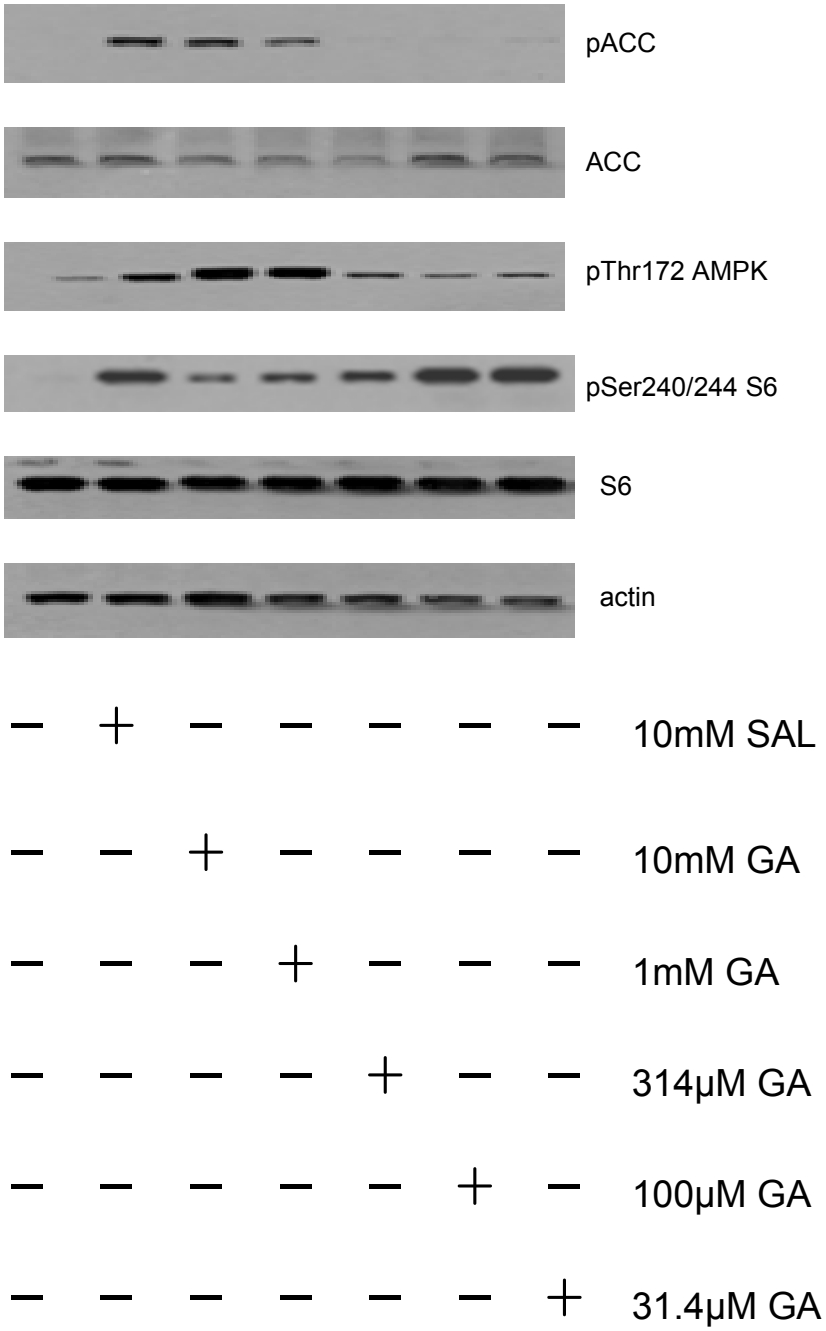


Fig 4.3: GA dose response on AMPK, ACC and S6 phosphorylation in H4IIE cells

H4IIE cells were serum starved for 2 hours, then treated with differing doses of GA for 3hrs followed by lysis and SDS-PAGE. Immunoblotting was carried out using two ACC antibodies- one which detects total ACC (ACC) and one which detects phosphorylated ACC (pACC), an antibody which detects AMPK only if it is phosphorylated on Thr172 (pThr172 AMPK), two S6 antibodies- one which detects total S6 (S6) and one which detects Ser240/244 S6 phosphorylation (pSer240/244 S6) and an actin antibody.10mM SAL is presented for comparison.

4.2.3 Time course of GA-dependent effects on AMPK, ACC and S6 phosphorylation

To determine the time course of GA-dependent effects on AMPK and S6 phosphorylation an experiment was carried out using western blot analysis (Fig 4.4).

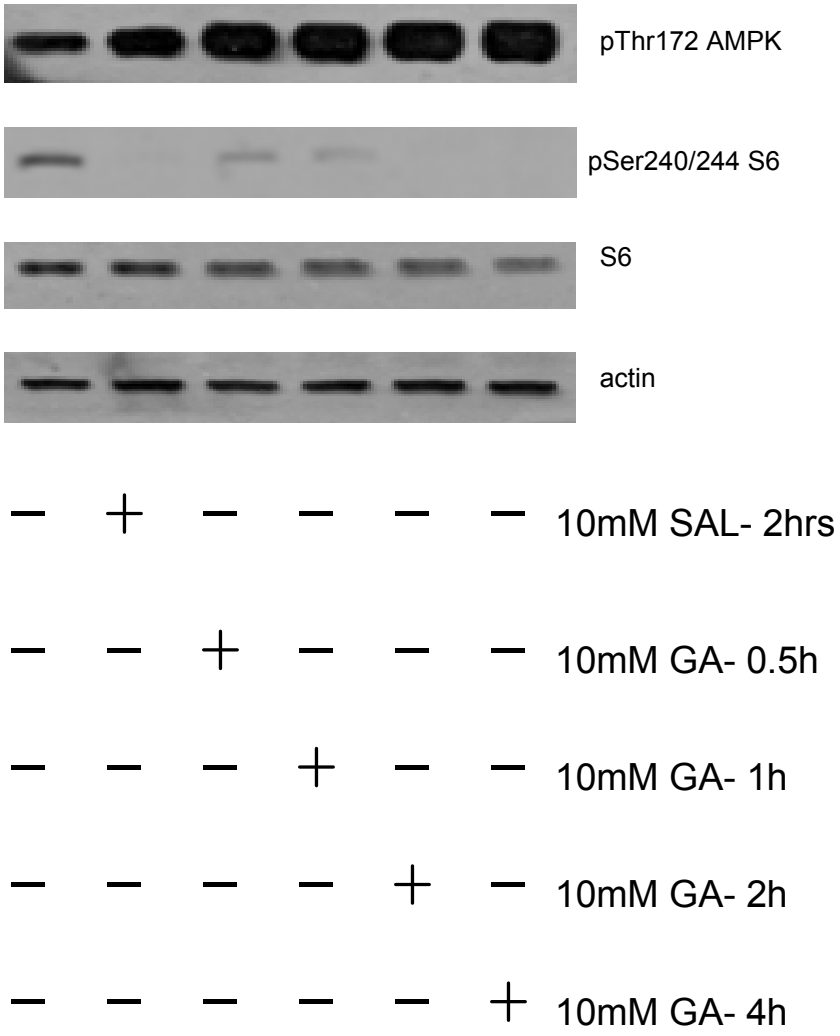


Fig 4.4: Time course of GA-dependent effects on AMPK and S6 phosphorylation in H4IIE cells

H4IIE cells were serum starved for 2 hours, then treated with 10mM GA in a time-course followed by lysis and SDS-PAGE. H4IIE cells were placed in serum-free medium for 2h, then treated with 10mM GA for 3hrs followed by lysis and SDS- PAGE. Immunoblotting was carried out using an antibody which detects AMPK only if it is phosphorylated on Thr172 (pThr172 AMPK), two S6 antibodies- one which detects total S6 (S6) and one which detects Ser240/244 S6 phosphorylation (pSer240/244 S6) and an actin antibody. 10mM SAL is presented for comparison.

The effect of GA on phosphorylation of AMPK was maximal at the shortest time point (0.5 h) but required two hours to exert its maximal effect on S6

dephosphorylation and these effects were sustained throughout the time course.

4.2.4 Effects of SAL: Dose response and time course.

To investigate the response of SAL more thoroughly, time course and dose-response experiments were carried out. The effect of SAL continued to increase up to 10mM, the highest dose that was tested. This is shown in Fig 4.5. For phosphorylation of ACC, AMPK and dephosphorylation of S6, effects were first noticeable between 2 and 5mM.

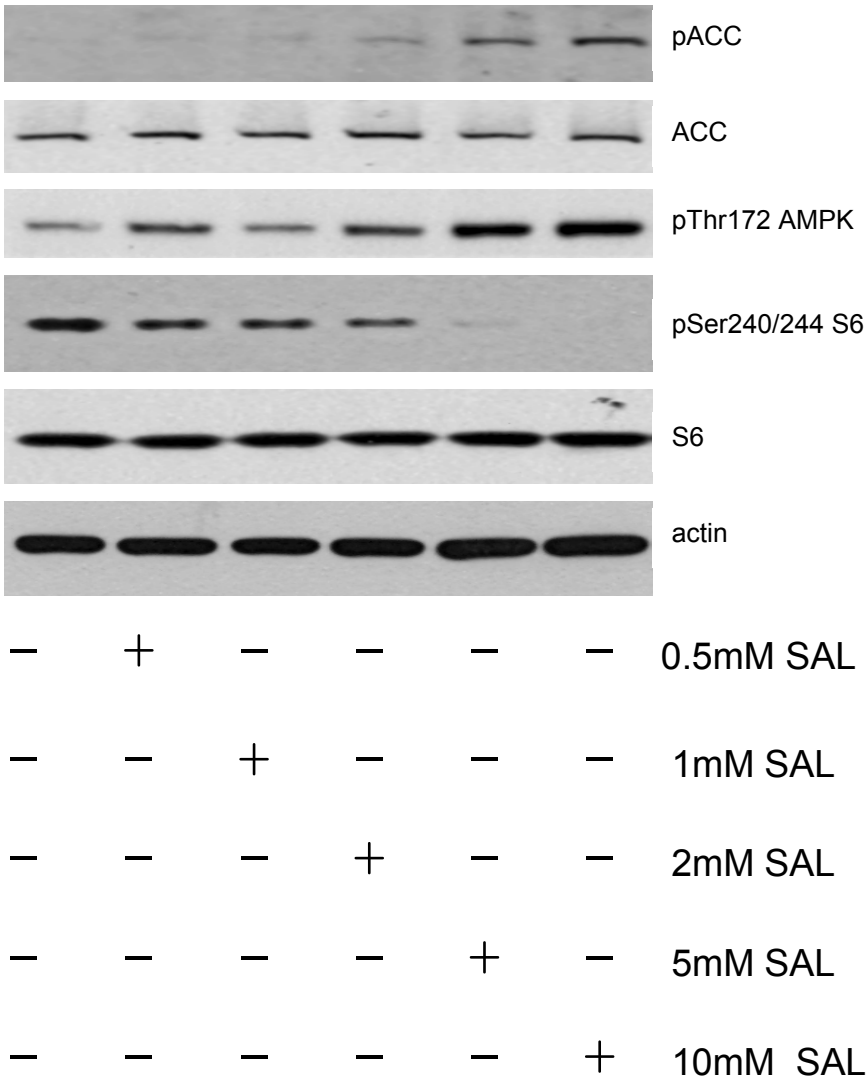


Fig 4.5: SAL dose response on AMPK, ACC and S6 phosphorylation in H4IIE cells
H4IIE cells were serum starved for 2 hours, then treated with differing doses of SAL for 3hrs followed by lysis and SDS-PAGE. Immunoblotting was carried out using two ACC antibodies- one which detects total ACC (ACC) and one which detects phosphorylated ACC (pACC), an antibody which detects AMPK only if it is phosphorylated on Thr172 (pThr172 AMPK), two S6 antibodies- one which detects total S6 (S6) and one which detects Ser240/244 S6 phosphorylation (pSer240/244 S6) and an actin antibody.

The maximal effect on phosphorylation of AMPK appeared not to occur until two hours had passed and maximal dephosphorylation of S6 took one hour (Fig. 4.6).

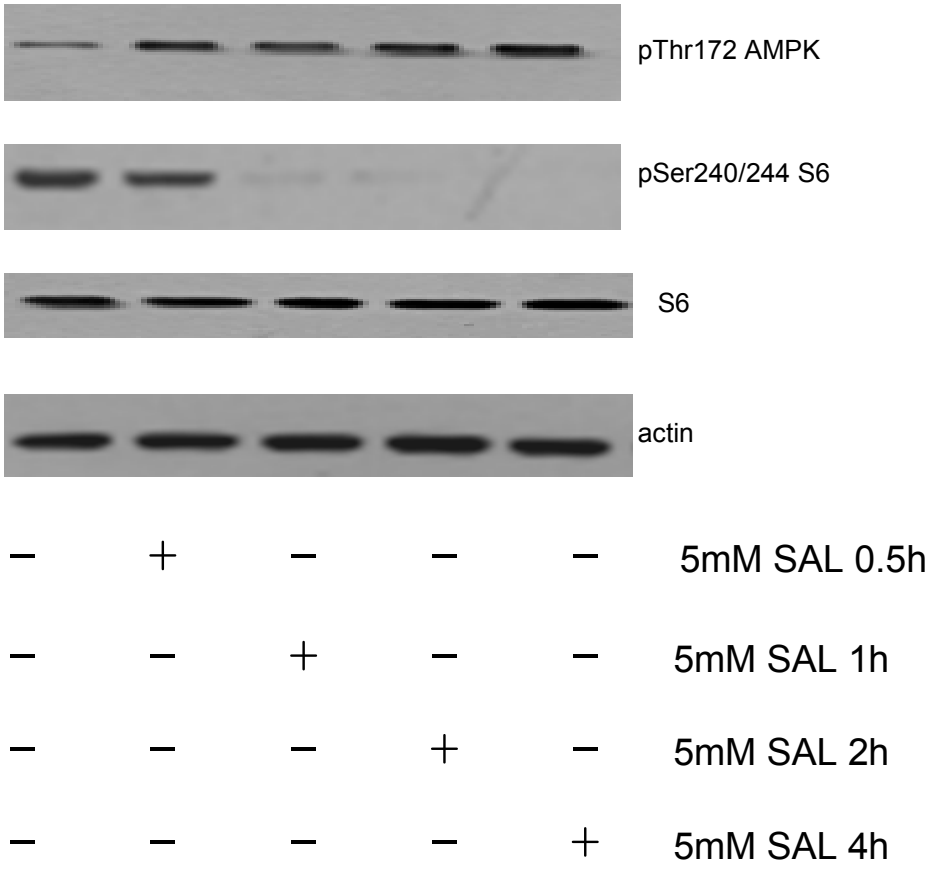


Fig 4.6: Time course of SAL-dependent effects on AMPK, ACC and S6 phosphorylation in H4IIE cells

H4IIE cells were serum starved for 2 hours, then treated with 5mM SAL for the times shown, followed by lysis and SDS-PAGE followed by lysis and SDS- PAGE. Immunoblotting was carried out using an antibody which detects AMPK only if it is phosphorylated on Thr172 (pThr172 AMPK), two S6 antibodies- one which detects total S6 (S6) and one which detects Ser240/244 S6 phosphorylation (pSer240/244 S6) and an actin antibody.

4.2.5 Comparison of SAL and acetyl salicylate

It was then decided to compare the effects of salicylate, which is not used clinically, with those of acetyl salicylate (aspirin), which is used clinically for its anti-inflammatory, analgesic, antipyretic and anti-thrombitic effects. These two structures are illustrated in Fig 4.7:

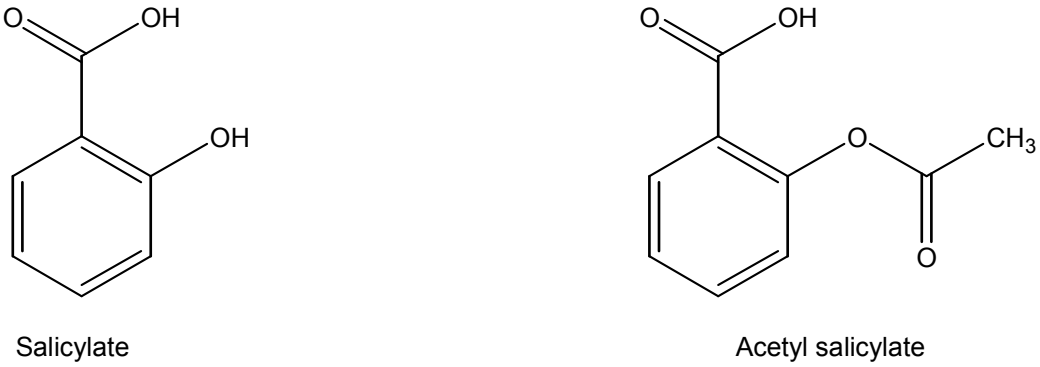


Fig 4.7: Structures of salicylate and aspirin

Western blotting experiments found that salicylate was much more effective than acetyl salicylate. This is shown in Fig 4.8:

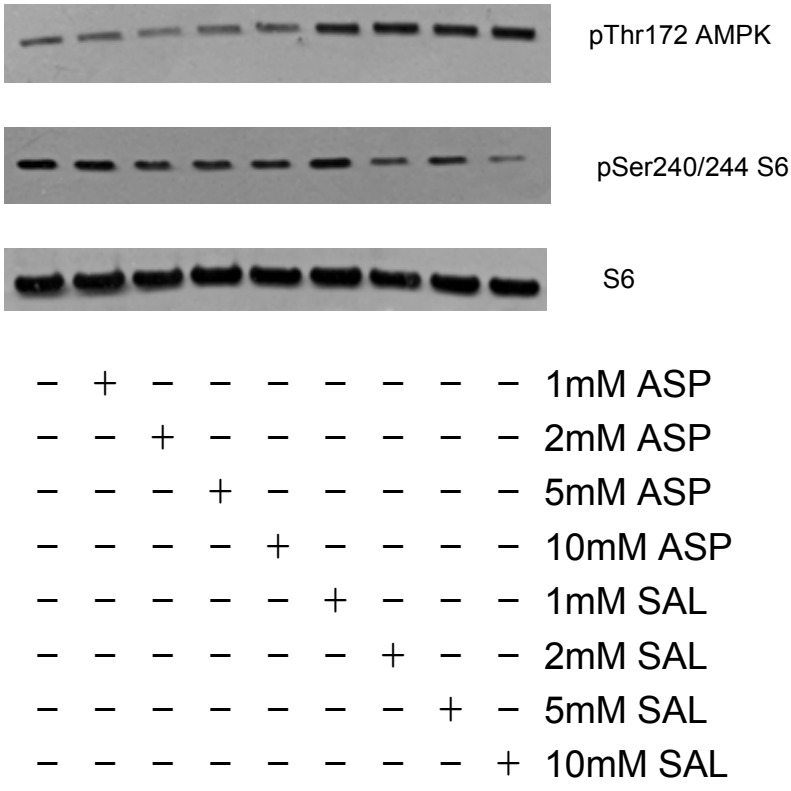


Fig 4.8: Comparison of SAL with aspirin

H4IIE cells were serum starved for 2 hours, then treated with differing doses of SAL and ASP for 3hrs followed by lysis and SDS- PAGE. Immunoblotting was carried out using an antibody which detects AMPK only if it is phosphorylated on Thr172 (pThr172 AMPK), 2 S6 antibodies- one which detects total S6 (S6) and one which detects Ser240/244 S6 phosphorylation (pSer240/244 S6).

4.2.6. Comparison of SAL with 2-methoxybenzoic acid

2-methoxybenzoic acid is an ester of methanol and salicylic acid. This is shown in Fig 4.9:

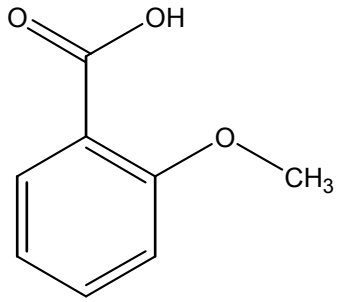


Fig 4.9: 2-methoxybenzoic acid

Cell culture and western blotting comparing these two compounds suggested that SAL was much more effective than 2-methoxybenzoic acid. We were unable to obtain a response to this latter compound that was any more than marginally different to basal levels of phosphorylation. This is shown in Fig 4.10:

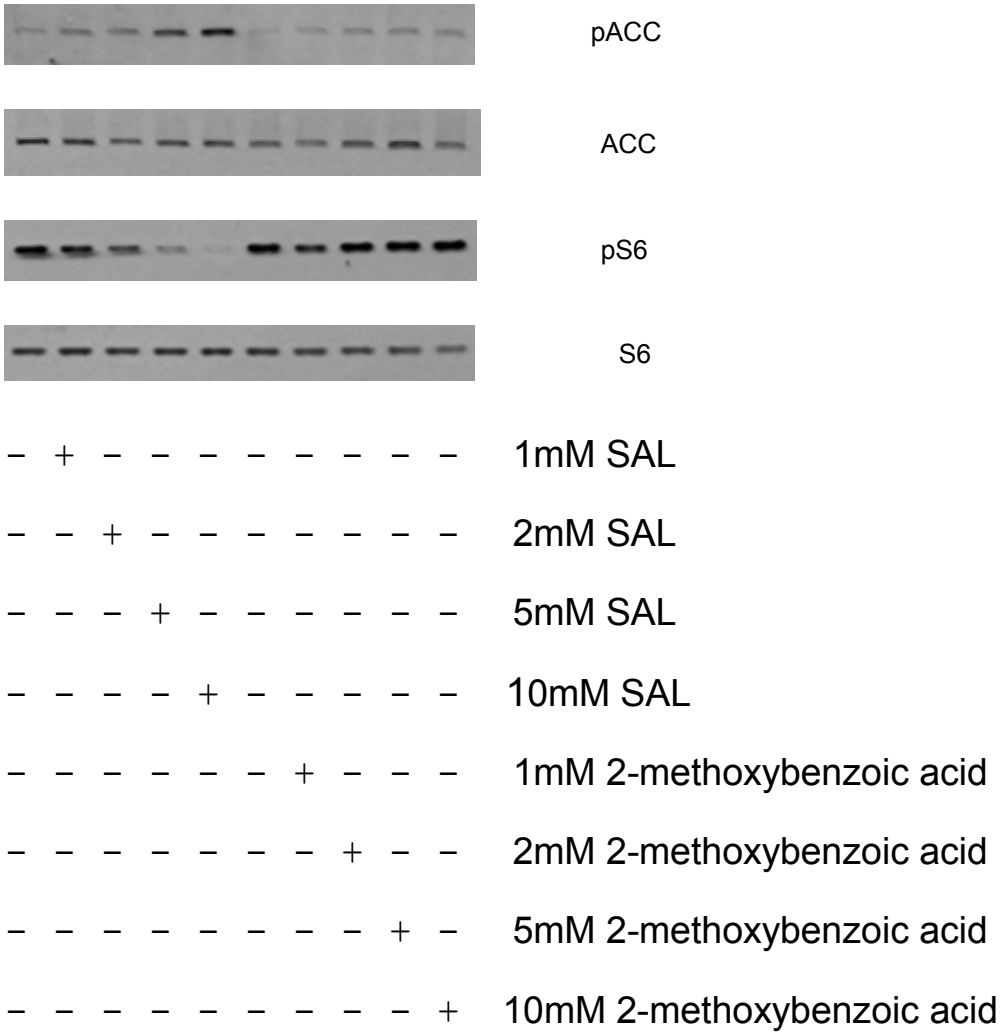
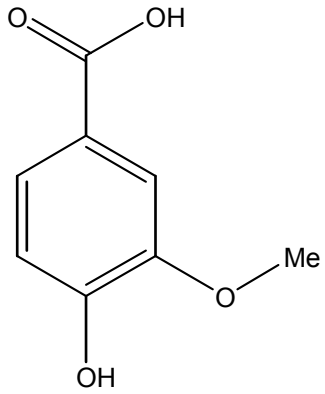


Fig 4.10: Comparison of SAL with 2-methoxybenzoic acid

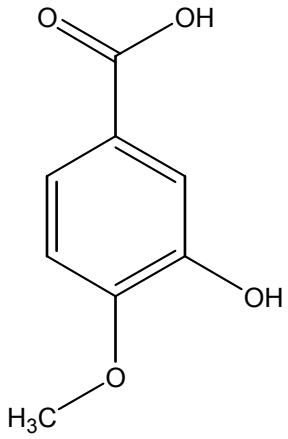
H4IIE cells were serum starved for 2 hours, then treated with differing doses of SAL and methyl ASP for for 3hrs followed by lysis and SDS-PAGE. Immunoblotting was carried out using two S6 antibodies- one which detects total S6 (S6) and one which detects Ser240/244 S6 phosphorylation (pSer240/244 S6) and an actin antibody.

4.2.7 Screening of further HBA analogues for effects on AMPK, S6 and ACC phosphorylation

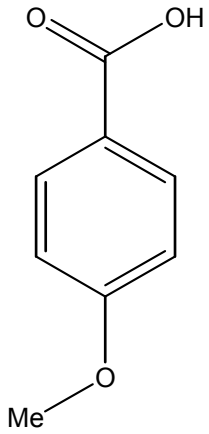
To further probe structure activity relationships, further screening was carried out, using additional HBA analogues that were mostly already available in the laboratory. The structures illustrated in fig 4.11 were screened.



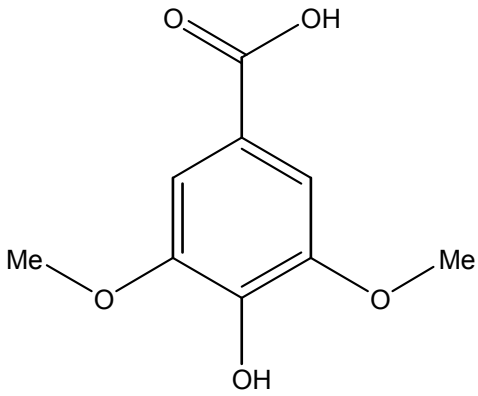
4 –OH-3-methoxy BA
(vanillic acid)



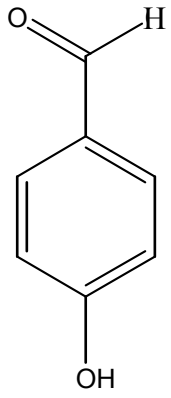
3 –OH–4-methoxy BA
(Isovanillic acid)



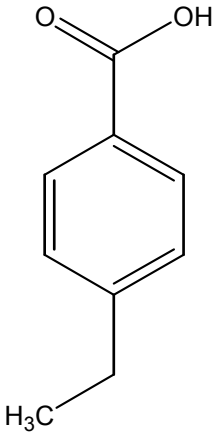
4 –methoxy-BA



3, 5–dimethoxy-4 -OH-BA
(syringic acid)



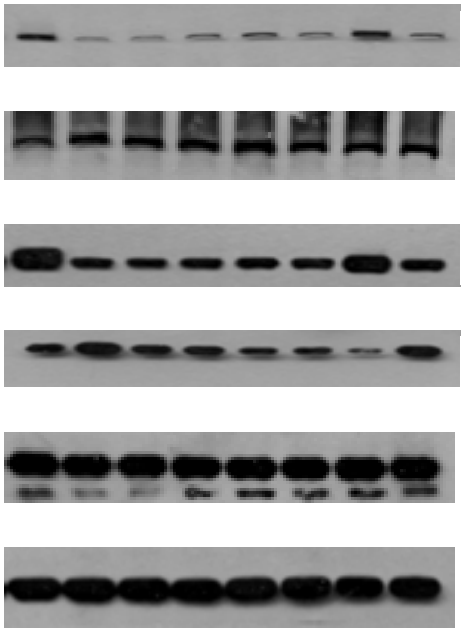
4 –OH-benzaldehyde



4–ethyl-BA

Fig 4.11: Further HBA analogues

The subsequent screen indicated that only 4–OH-benzaldehyde induced effects on AMPK, ACC and S6 that are comparable with GA, which was used as a positive control. This is shown in Fig 4.12.



+	−	−	−	−	−	−	−	GA (positive control)
−	−	+	−	−	−	−	−	4-methoxy-BA
−	−	−	+	−	−	−	−	3, 5 dimethoxy- 4-OH-BA (syringic acid)
−	−	−	−	+	−	−	−	3-OH-4-methoxy-BA (isovanillic acid)
−	−	−	−	−	+	−	−	4-ethyl-BA
−	−	−	−	−	−	+	−	4-OH-benzaldehyde
−	−	−	−	−	−	−	+	4-OH-3-methoxy-BA

Fig 4.12: Investigation of further benzoic acid analogues

H4IIE cells were serum starved for 2 hours then treated with 5mM of the benzoic acid analogues shown above for 3hrs followed by lysis and SDS-PAGE. Immunoblotting was carried out using two ACC antibodies- one which detects total ACC (ACC) and one which detects phosphorylated ACC (pACC), an antibody which detects AMPK only if it is phosphorylated on Thr172 (pThr172 AMPK), two S6 antibodies- one which detects total S6 (S6) and one which detects Ser240/244 S6 phosphorylation (pSer240/244 S6) and an actin antibody.

4.2.8 Dose response effects of 4–OH–benzaldehyde on AMPK, ACC and S6 phosphorylation

A concentration assay experiment found that 4–OH–benzaldehyde reached its maximum effect at around 3-5 mM with respect to ACC and AMPK phosphorylation but S6 phosphorylation was undetectable at 2 mM. (Fig 4.13).

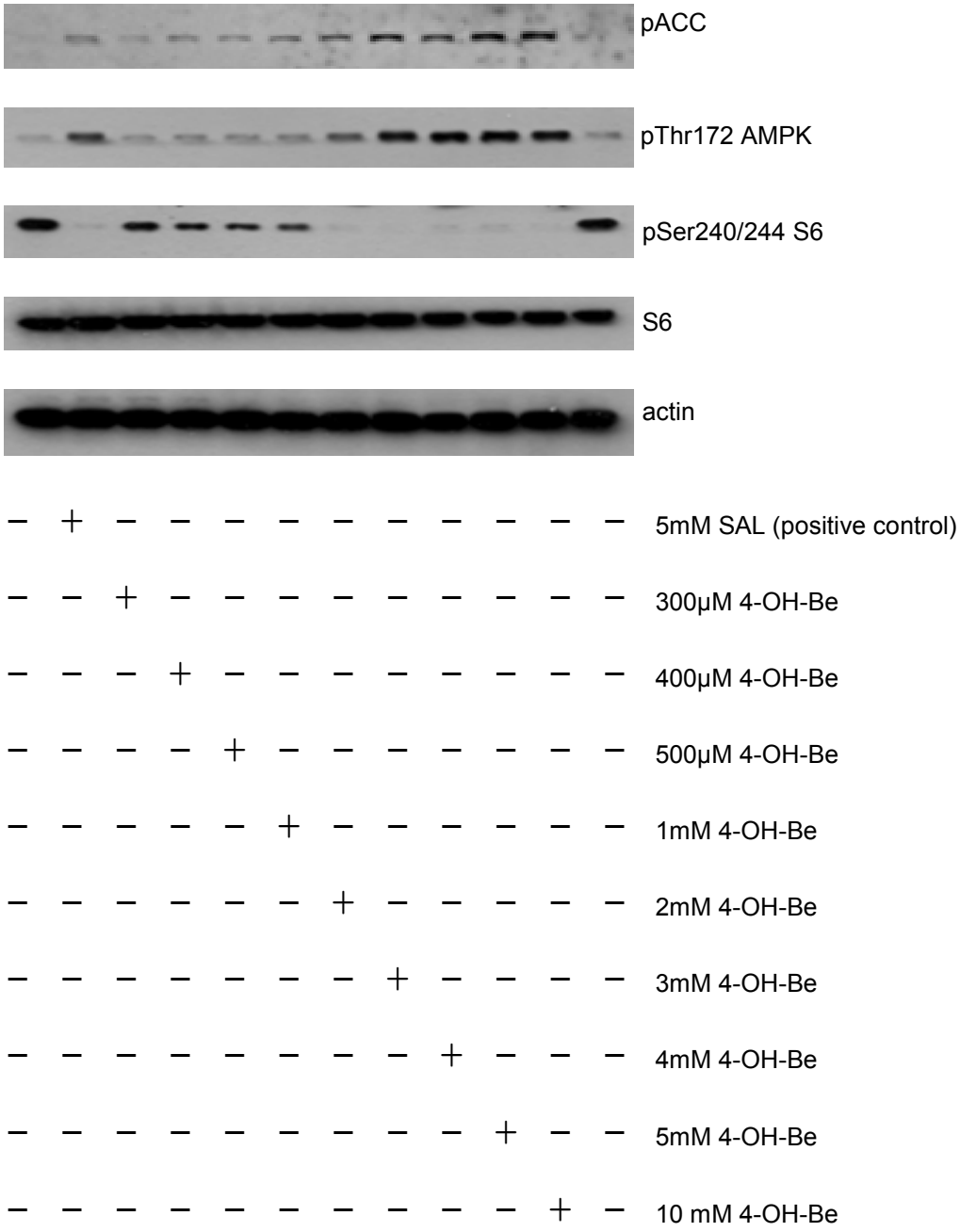


Fig 4.13 Dose response of 4-OH benzaldehyde dependent effects on AMPK, ACC and S6 phosphorylation in H4IIE cells

H4IIE cells were serum starved for 2 hours then treated with differing doses of 4-OH benzaldehyde shown above for 3hrs followed by lysis and SDS-PAGE. Immunoblotting was carried out using one ACC antibody that detects phosphorylated ACC (pACC), an antibody which detects AMPK only if it is phosphorylated on Thr172 (pThr172 AMPK) two S6 antibodies- one which detects total S6 (S6) and one which detects Ser240/244 S6 phosphorylation (pSer240/244 S6) and an actin antibody.

4.2.9 Time course of AMPK and S6 phosphorylation in response to 4–OH–benzaldehyde

In a time-course at 5 mM, it appears that with respect to phosphorylation of ACC and AMPK, 4-OH-benzaldehyde induced phosphorylation within 30 minutes but this seemed to diminish at later time points. However complete dephosphorylation of S6 took two hours. This is shown in Fig 4.14. It was also noted earlier that 4-OH-BA was ineffective suggesting the benzaldehyde moiety is necessary for this effect.

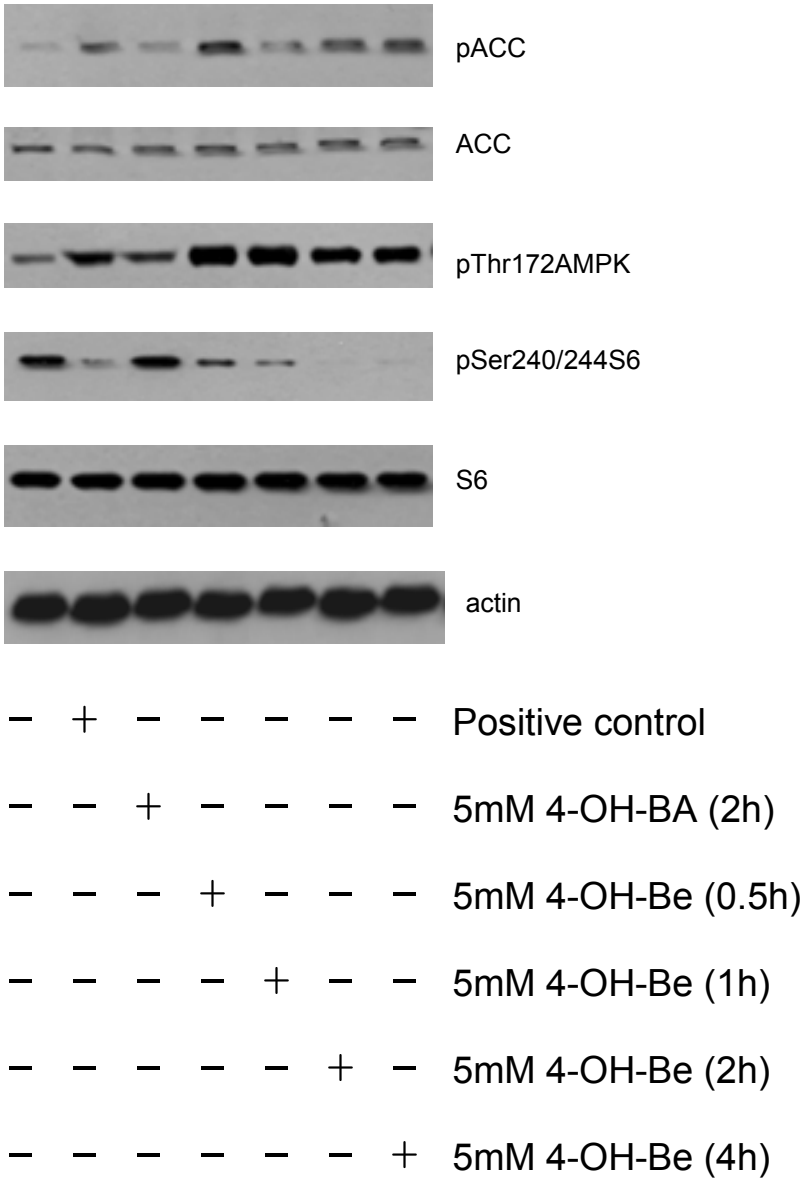


Fig 4.14: Time course of 4-OH benzaldehyde dependent effects on AMPK, ACC and S6 phosphorylation in H4IIE cells

Serum starved H4IIE cells were treated as above for 3hrs followed by lysis and SDS-PAGE. Immunoblotting was carried out using two ACC antibodies- one which detects total ACC (ACC) and one which detects phosphorylated ACC (pACC), an antibody which detects AMPK only if it is phosphorylated on Thr172 (pThr172 AMPK) two S6 antibodies- one which detects total S6 (S6) and one which detects Ser240/244 S6 phosphorylation (pSer240/244 S6) and an actin antibody.

4.2.10 Investigation of the role of the Fenton reaction in effects of benzoic acids on AMPK, ACC & S6 phosphorylation.

Some phenolic compounds are unstable in the presence of oxygen and metal ions because of the Fenton reaction (Long *et al*, 2000). This may be significant in cell culture because cells are cultured in pro-oxidant conditions and ferric nitrate is present in the cell culture medium at 0.248 μM. Ferric iron can reduce molecular oxygen to produce superoxide:

$$Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O^-_2$$

Superoxide reacts with groups on the polyphenol to generate hydrogen peroxide and oxygen radicals which reduce molecular oxygen and initiate a series of redox reactions:

$$O^-_2 + 2OH \longrightarrow H_2O_2 + O_2$$

The hydrogen peroxide produced *in vivo* would be limited due to high levels of catalase in the liver but under cell culture conditons in the absence of catalase it could influence biochemical pathways necessary for emergency responses. Whether or not such responses were physiologically relevant would require further investigation. We had noted that the addition of gallic acid to the cell culture medium caused the medium to darken. This is likely to be due to complexation with iron in the medium, as iron is known to bind gallic acid to form deeply coloured complexes (Miller, 1996). This is illustrated in Fig 4.15 where the colour difference between DMEM and 10mM GA DMEM can be compared.

Fig 4.15: The change in colour of DMEM caused by GA

To investigate any potential interaction of BAs with metals more quantitatively, the absorbance spectra from wavelengths of 300-800 nm were obtained from samples containing 40μM BA and 40μM metal ion. These results are shown in Table 4.1, presented alongside data on AMPK regulation (this thesis) and data on uncoupling from a previous study.

		Compound used												
		0	BA	SAL	3-HBA	4-HBA	2,3-HBA	2,4-HBA	2,5-HBA	2,6-HBA	3,4-HBA	3, 5-HBA	GA	4-Be
Transition metal used	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Fe^{2+}	0	0	0	0	0	0.739	0	2.760	0.393	0.056*	0	0.331	+3
	Fe^{3+}	0	0	0	0	0	0.707	0	2.764	0.386	0101*	0	0.323	0
	Cu^{2+}	0	0	+3	0	0	0.309	0	2.740	0.361	0	0	0.391	+3
		AMPK activation (this thesis)												
		-	-	+	-	-	-	-	-	-	-	-	+	+
		Uncoupling properties of compound, (Brody, 1956)												
		0	0	98	ND	ND	55	9	7	10	ND	ND	ND	ND

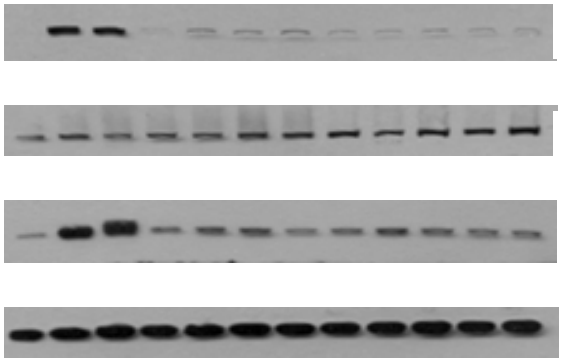
Table 4.1: Measurement of metal-binding of HBAs and related compounds.
This data shows absorbance measured in absorbance units (AU). All absorbance maxima detected were at 340 nm apart from those highlighted by an asterisk* which were detected at 567 nm. Values >3 are highlighted in red. However, the samples were dissolved in UPW for this experiment to facilitate spectrophotometry and the pH of this was found to be 5.23, compared to a pH of 7.4 in the case of DMEM, therefore it is unclear to what extent pH influences metal binding and ionisation compared to physiological events in this case. For comparison, the ability of each compound to activate AMPK phosphorylation and uncouple mitochondria are shown where this data was available, presented as % uncoupling of control (Brody, 1956). Each value is the mean absorbance of three different readings.

In summary, no relationship was confirmed between iron binding and activity as salicylate, which did induce phosphorylation of ACC and AMPK and dephosphorylation of S6, did not bind iron.

Oxidation state would appear to have limited effect on these results as there was very little differences in results between Fe^{2+} and Fe^{3+} apart from in the case of 4-OH-benzaldehyde.

It was also noted that although copper-binding was a common property amongst the compounds activating ACC, AMPK and dephosphorylation of S6, it cannot be sufficient because some inactive compounds were also found to bind copper.

To test the role of the extracellular Fenton reaction in mediating the effects of GA, SAL and other hydroxybenzoic acids, the medium was substituted for normal saline which does not contain any iron. These results are shown in Fig 4.16. In comparison with earlier experiments carried out in the presence of DMEM, the results indicate that GA and SAL do not require extracellular iron to mediate their effects on AMPK, ACC and S6.



−	+	−	−	−	−	−	−	−	−	−	−	2-hydroxy BA (salicylate)
−	−	+	−	−	−	−	−	−	−	−	−	3, 4, 5-trihydroxy BA (gallic acid)
−	−	−	+	−	−	−	−	−	−	−	−	BA
−	−	−	−	+	−	−	−	−	−	−	−	3-hydroxy BA
−	−	−	−	−	+	−	−	−	−	−	−	4-hydroxy BA
−	−	−	−	−	−	+	−	−	−	−	−	2, 3-dihydroxy BA
−	−	−	−	−	−	−	+	−	−	−	−	2, 4-dihydroxy BA
−	−	−	−	−	−	−	−	+	−	−	−	2,5-dihydroxy BA (gentisate)
−	−	−	−	−	−	−	−	−	+	−	−	2, 6-dihydroxy BA
−	−	−	−	−	−	−	−	−	−	+	−	3, 4-dihydroxy BA
−	−	−	−	−	−	−	−	−	−	−	+	3, 5-dihydroxy BA

Fig 4.16: Effect of benzoic acids on AMPK and ACC phosphorylation in H4IIE cells: incubation in normal saline

H4IIE cells were incubated in normal saline followed by treatment with 10mM of the benzoic acids shown for 3hrs followed by lysis and SDS-PAGE. Immunoblotting was carried out using two ACC antibodies- one which detects total ACC (ACC) and one which detects phosphorylated ACC (pACC), an antibody which detects AMPK only if it is phosphorylated on Thr172 (pThr172 AMPK), and an actin antibody.

4.3 Discussion

4.3.1 Structural requirements for effects of benzoic acids and related compounds on AMPK, ACC and S6.

Of all the benzoic acids screened, only GA and SAL induced ACC and AMPK phosphorylation and inhibited S6 dephosphorylation, although other HBAs induced partial effects on S6 alone. Both GA and SAL possess hydroxyl groups and a comparison of SAL with either 2-methoxybenzoic acid or acetyl salicylate showed SAL to be active but these analogues to be inactive, confirming that the presence of a free 2-OH group was required for the effect. This correlation is not upset by the anti-hyperglycaemic properties of aspirin observed *in vivo,* as these are thought to depend on the drug's conversion to SAL, which does not occur in cell culture (Hawley, 2012). However, differing hydroxylation positioning of the two molecules suggests that it might be unlikely that they interact with a single target. Equally, there were many hydroxybenzoic acids that were inactive at the concentrations tested, suggesting that hydroxylation by itself might be necessary but is not sufficient to trigger effects on AMPK and S6 phosphorylation. The third active compound in the screen was 4-OH-benzaldehyde. A comparison of 4-OH-BA and 4-OH-benzaldehyde confirmed that while 4-OH-benzaldehyde was active, 4-OH-BA was not, indicating that the acid moiety is dispensible. In addition, the hydroxyl groups in the three active compounds (SAL, GA and 4-OH-benzaldehyde) were in different locations on the ring. Thus the relationship between structure and function does not yet appear to be straightforward. It is important to bear in mind therefore that the molecules under investigation are so simple that there may be more than one target

(and therefore more than one chemical motif) mediating the effects. This will obviously make structure/function studies more difficult to interpret.

Effects on S6 were usually but not always accompanied by effects on AMPK and ACC. We did not establish whether or not effects of drugs on S6 in the absence of AMPK depend on kinase inhibition or phosphatase activation, or both, but clarification of this would possibly begin to unravel the mechanism underlying these effects.

4.3.2 Functional requirements: Compounds that regulate AMPK, ACC and S6 inhibit intracellular ATP production

As discussed in the introduction, two competing mechanisms for the antihyperglycaemic effects of HBAs have emerged, focussed mostly on studies of SAL. These mechanisms are inhibition of gluconeogenesis, through inhibition of mitochondrial ATP production and anti-inflammatory effects of the drugs. The data from our panel of HBAs suggests that it may be possible to differentiate between these two competing models. This is because SAL, 2,5 di-OH-BA and 2,6 di-OH-BA have each been reported to possess anti-inflammatory properties (Meyer, 1948) but amongst these only SAL is anti-hyperglycaemic and induces AMPK activation (Smith, 1952, Mahler 1960, Hawley, 2012), suggesting that AMPK activation rather than anti-inflammatory effects of HBAs is a better reporter of antihyperglycaemic effects of HBAs.

The effects of the HBAs on AMPK can probably be directly attributed to their uncoupling properties, as both SAL and GA but not other HBAs are effective uncouplers at the concentrations used (Nakagawa *et al*, 1995; Brody and Fouts 1956; Meade, 1954; Whitehouse and Dean 1965). While this thesis

was in preparation, direct effects of SAL on AMPK were also reported (Hawley *et al* 2012) but this paper also ruled out a role for AMPK in anti-hyperglycaemic effects of SAL. Taken together with the data in this thesis, a possible model emerges of AMPK-independent direct effects of mitochondrial inhibition on hepatic glucose production (still to be measured), with the anti-inflammatory effects of the HBAs insufficient to support by themselves these antihyperglycaemic responses. Further work will be required to substantiate this analysis and to understand more clearly the activity of GA and 4-OH-benzaldehyde, whose effects on cells have been studied less extensively than the other compounds discussed.

4.3.3 Role of metal-binding

Many BAs are known to bind to metals and a preliminary investigation was carried out to assess whether or not this is likely to be important in the properties of these drugs, as earlier studies on metformin analogues suggested that binding of this drug to copper ions correlated closely with cellular responses (Logie *et al,* 2012). Spectrophotometric analysis in this thesis suggested that activity of BAs correlated more closely with copper binding than iron; however, some BAs that bind either of these metals had no significant effects on the cellular responses under investigation. In additional experiments, we excluded any role of iron in the DMEM. Preliminary studies were also carried out using specific copper and iron-sequestering drugs including triethylaminetetramine to bind copper and desferoxamine to bind iron. The aim of these experiments was to determine more directly whether or not the cellular effects we were studying depend on metal-binding of BAs

but it was impossible to draw firm conclusions on the data available. Further work will be required to establish whether or not metal binding is necessary for the action of these drugs on AMPK but even if metal-binding is required, the results so far indicate that it is not sufficient.

Whether any involvement of metals is due to inhibition of specific metalloenzymes would also require further study. The possibility of induction of intracellular ROS through the Fenton type reaction described earlier remains. However, it seems unlikely that the HBA derivatives studied here would differ significantly in their susceptibility to this reaction. Further study to determine the stability of each of these compounds *in vivo* may help to clarify as to whether this is a possibility.

4.3.4. Physiological relevance

The concentrations of SAL that inhibit the mitochondria are pharmacologically-relevant but whether or not the effects of GA are relevant to dietary levels will require further study. Our work with hepatocytes found that these cells certainly respond to GA but GSE also contains a large amount of galloylated PACs that may be more active because hydrophobic alkyl esters of GA, which are more hydrophobic than GA itself, are also more potent inhibitors of ATP production (Nakagawa *et al*, 1995). However, a study by Cacetta *et al* (2000) showed that 2-4 hours after ingesting 5ml/kg body weight red wine containing 9.5mg/ml GA plasma levels were 1.8μM (Cacetta *et al,* 2000). In this study concentrations over 1mM were required to cause the physiological response observed therefore further *in vitro* study including lower concentrations over a longer time period may be of benefit.

CHAPTER 5- CELLULAR RESPONSES TO LINGONBERRY AND CRANBERRY EXTRACTS

5.1 Introduction

Besides grape seed and pine bark, PACs are also found in berries such as *Vaccinium oxycoccus* (cranberry) and *V.vitis-idaea* (lingonberry).

A-type and B-type PACs have characteristically different structures (Fraga *et al,* 2010) and have different three dimensional shapes (Porter, 1993) which may affect their ability to interact or bind with receptors e.g. on the plasma membrane. Berry polyphenols including PACs have been shown to inhibit pancreatic amylase (Grussu *et al*, 2011) and PACs have been implicated in the inhibition of pancreatic lipase (McDougall *et al*., 2009; Kimura *et al*, 2011). Inihbition of amylase could influence starch digestion and therefore has been proposed as a potential prophylactic measure with respect to post prandial hyperglycemia (Grussu *et al*, 2011). It has been suggested that the presence of larger PACs (Kimura, 2011) and procyanidins (Sugiyama *et al*, 2007) are associated with increased lipase inhibition *in vitro* (Kimura *et al*, 2011) and *in vivo* (Sugiyama *et al*, 2007) but also that A type PACs inhibit pancreatic lipase more effectively than B type (Kimura, 2011)

5.1.1 Cranberry

V.oxycoccus has been divided into various subspecies. American cranberry (*V. macrocarpon)* is the most extensively commercially exploited of these. Cranberry is native to North America and found in Newfoundland, Minnesota, Illinois, Ohio, Indiana, Tennessee and Carolina. It grows in bogs and

swamps in acidic soil (Vander Kloet, 1988). In the 1820s cranberries were shipped to Europe (Cape Cod Cranberry Growers' Association) and by 1923 were observed growing wild in Ireland (Stelfox, 1923).

Cranberry has been suggested to be beneficial in the treatment of urinary tract infections (Avorn *et al*, 1994), type 2 diabetes (Kotiokari *et al*, 2002) and cancer (Yan *et al*, 2002). A study by Avorn *et al* in 1994 suggested cranberry juice to have bacteriostatic properties, possibly contributed to by adhesion inhibition. It was also noted that the beneficial effects of cranberry juice were observed after one to two months, perhaps compatible with the time frame required for modification of gut bacteria. Bacterial cell adhesion has been shown to be inhibited by cranberry juice *in vitro,* by inhibiting attachment of bacterial fimbriae to cells expressing oligosaccharide receptors similar to those on uroepithelial cells (Foo *et al*, 2000). Therefore it has been suggested that the apparent prevention and treatment of urinary tract infections by cranberry juice may be due to its modification of gut flora and anti-adhesive properties respectively.

Cranberry has also been suggested to elevate high density lipoprotein-cholesterol levels in a patient based study using 31 healthy sedentary adult males, showing an 8% increase (Ruel *et al*, 2006) compared to a 10% increase noted by fibrates in a previous meta-analysis (Birjmohun *et al*, 2005). Flavan-3-ols and procyanidins have been proposed as potential antihypertensive agents by inhibition of angiotensin converting enzyme (ACE) (Actis-Goretta *et al*, 2003).

Cranberry has also been found to improve the lipid profile of patients with T2D (Lee *et al*, 2008). In one study on rats fed a high fructose diet, metabolic

abnormalities appeared to be ameliorated when the diet was supplemented with 3% cranberry pomace. The animals on the supplemented diet had lower fasting plasma insulin, cholesterol and triacylglycerol levels as well as decreased insulin resistance (Khanal *et al*, 2010).

Cranberry has also been proposed to have antiproliferative effects in lung H460), cervical (ME180), prostate (PC3), breast (MCF- 7), colon (HT- 29) and leukaemia (K562) cell lines (Yan, 2002). Another study found that antiproliferative effects in oral (KB; CAL27), colon (HT- 29; HCT116; SW480; SW620) and prostate (RWPE- 1; RWPE- 2; 22Rv1) cancer cell lines are enhanced when polyphenolic rich extracts were used (Seeram, 2004). Hydroxycinnamates have been suggested to be the active agents with respect to breast, cervical and prostate cell lines (Murphy *et al*, 2003).

5.1.2 Lingonberry

One subspecies of *V.vitis-idaea* exists - *V.vitis-idaeaminus* which differs from the main species only in length of the leaves and size of the berries (United States Department of Agriculture). Both are suitable for commercial use. Like cranberry, lingonberry is also native to North America and found in Greenland, Connecticut and the Aleutian Islands. It is also found in Scandinavia and Russia (Ek *et al*, 2006). It grows in raised bogs, lichen woodlands, heaths, high moors, tundra, cliffs and mountain summits (Vander Kloet, 1988)

Lingonberry has been suggested to be effective in the treatment of cardiovascular disease (Kivomaki *et al*, 2011) and urinary tract infection (Kotiokari *et al*, 2002). Lingonberry juice has been shown to increase

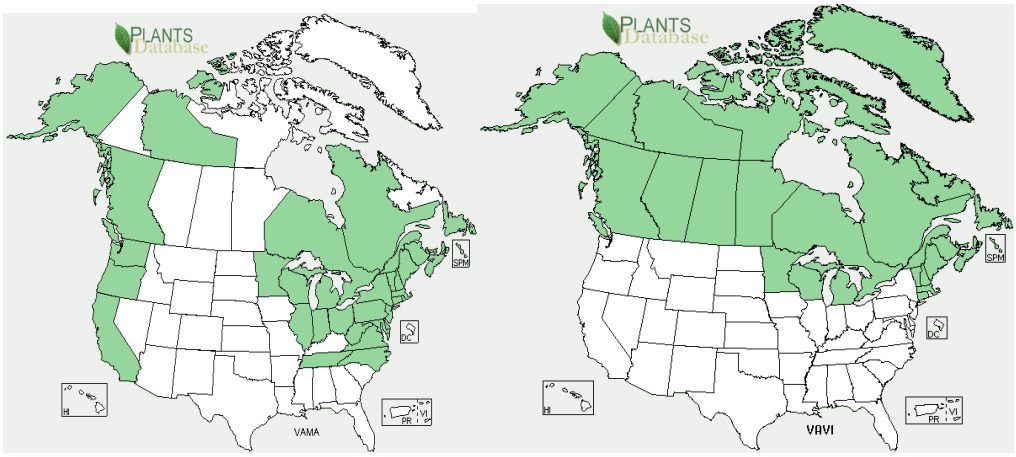
endothelial dependent vasodilation in hypertensive rats (Kivimaki *et al*, 2011) and has been proposed as a potential treatment (Tapiainen *et al*, 2012) and preventative measure (Kotiokari *et al*, 2002) for urinary tract infections. It has been found to reduce proliferation in colon and breast cancer cell lines. This effect correlated with vitamin C levels in the sample but was not observed in the presence of ascorbate alone, suggesting a possible synergistic effect between vitamin C and other components of the sample (Olsson *et al*, 2004).

5.1.3 Molecular basis for cellular effects.

The molecular targets underlying responses to polyphenols are not well understood. Various attributes of polyphenols have been proposed to contribute to their apparent medicinal effects, including antioxidant capacity (Zheng *et al*, 2003; Shyur *et al*, 2005), when measured in plants used for medicinal effect. Prevention of ischaemia- reperfusion injury by inhibition of nitric oxide synthase has also been observed (Brouet and Ohshima, 1995). Cell culture studies have shown cranberry anthocyanins to reduce hydrogen peroxide damage, to inhibit oxidation of cell membrane lipids and to upregulate inflammatory mediators interleukin- 8 (IL8), monocyte chemotactic protein- 1 (MCP-1) and intracellular adhesion molecule (ICAM-1), as these molecules are involved in the recruitment of leucocytes to areas of endothelial damage. These studies were performed using human microvascular endothelial cells (Youdim *et al*, 2002).

5.1.4 Geographical distribution

The geographical distribution of these plants in the USA is shown in Fig 5.1. Green indicates the presence of the relevant plant. Images are from the United States Dept of Agriculture Plants database.



Cranberry Lingonberry

Fig 5.1: Geographic distribution of cranberry and lingonberry

This data suggests cranberry to grow in wet areas and lingonberry in cold, dry areas (Images from United States Dept of Agriculture)

5.1.5 Proanthocyanidins

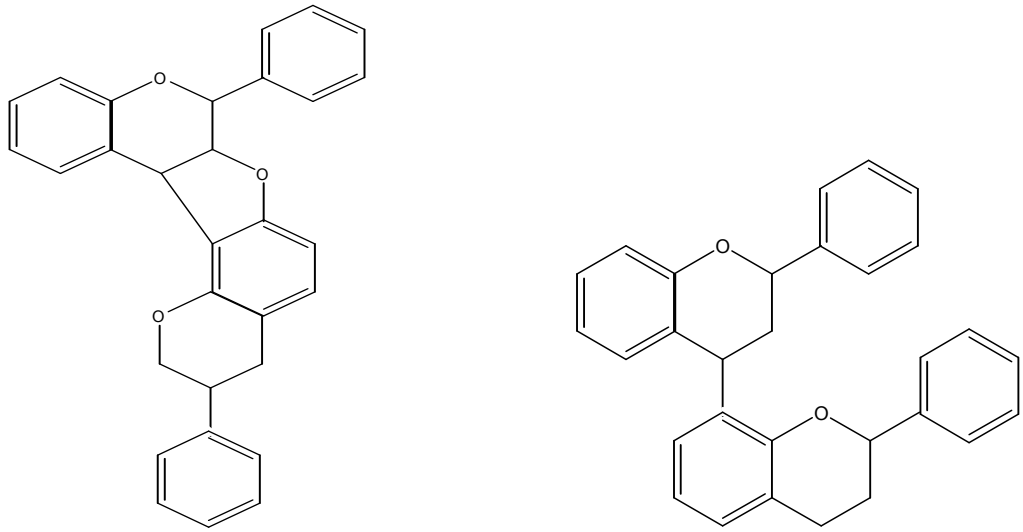
Proanthocyanidins, also called condensed tannins (Hagerman, 2011), are widespread in nature and are reported to be the most ingested class of polyphenol as they are found at reasonable levels in a wide range of foodstuffs as shown in Table 5.1:

Food	PAC	With peel	No peel	White	Rose	Red	Black	Baking	Dark	Milk
Beer	2									
Wine				1	2	62				
Apple juice	12									
Apples		43-136	17-99							
Apricots		11.32								
Babyfood	0-54									
Chocolate								1636	234	152
Bananas	3									
Barley	99									
Beans	0-767									
Berries		24-664								
Cherries		19								
Cocoa powder	1373									
Coffee	0.1-3.7									
Cranberry juice	22									
Currants						5	158			
Dates	11									
Grape juice	49									
Grape seeds	373									
Grape skins	48									
Grapes	62-83									
Kiwi fruit	3-14									
Mango	13									
Nectarines	22-29									
Nuts	0-501									
Peaches		30-72								
Peanuts	16									
Pears	32-42									
Plums	28-247									
Pomegranate	1									
Spices	742-8137									

Table 5.1: PAC concentration of various foodstuffs. Units are mg/ 100g (US Dept of Agriculture, 2004).

PACs can be found as dimers to polymers (DP or degree of polymerisation from 2) upwards of flavan-3-ol units, such as epicatechin, which can be joined by two type of linkage. A type PACs contain at least one doubly linked unit with C4-C8 and C2–O7 bonds and B type PACs are singly linked through a C4–C8 bond. The additional bond in A type PACs causes the loss

of 2 hydrogen atoms, reducing the molecular weight by 2. These structures are illustrated in Fig 5.2:



Type A Type B

Fig 5.2: Schematic Illustration of Different Types of PACs

Examples of A-type and B-type proanthocyanidins (PACs). A-types have doubly linked units and B-types are singly linked.

5.1.6 Anthocyanidins

Proanthocyanidins yield anthocyanidins upon oxidative cleavage as shown in Fig 5.3: (Hagerman, 2011): This has been used as a diagnostic test for PACs (Porter, 1993) and is the reason behind their name.

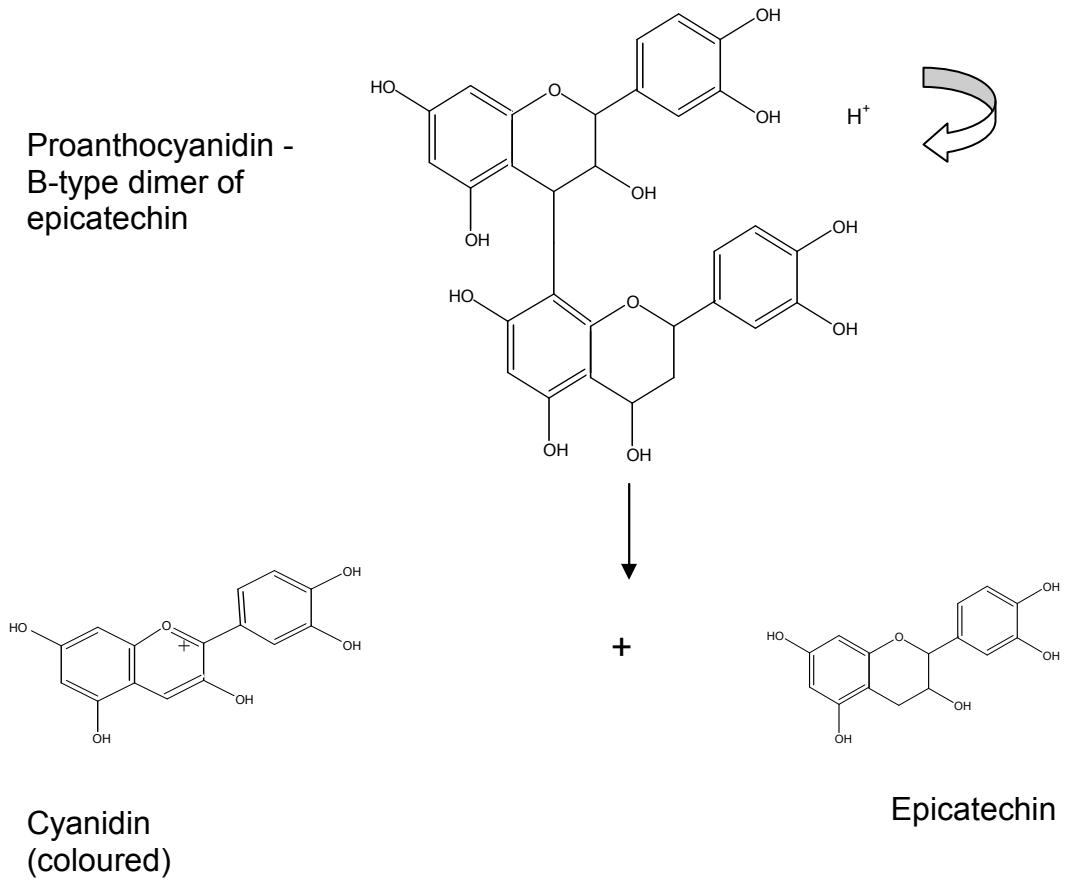


Fig 5.3: Hydrolysis of proanthocyanidins

Hydrolysis of this PAC dimer yields one anthocyanidin and one catechin whereas a PAC trimer would yield 2 anthocyanidins and one catechin and so on (Hagerman, 2011).

5.1.7 Anthocyanins

Anthocyanins are glycosylated anthocyanidins. The basic structure is shown in Fig 5.4 (Vermerris and Nicholson, 2006):

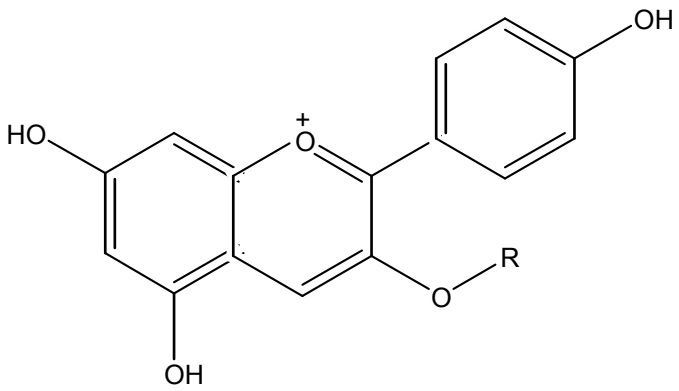


Fig 5.4: Structure of pelargonidin-3-glucoside, one of the anthocyanins
Anthocyanins are glycosylated anthocyanidins. R= glycosyl group

Anthocyanins are the compounds which give berries their red or blue colour and these have been suggested to have anti-inflammatory, antioxidant and vasoprotective effects (Wada and Ou, 2002). In addition, a study by Tsuda *et al,* (2003) suggested anthocyanins ameliorated weight gain and hyperglycemia caused by a high fat diet. This conclusion is undermined because the high fat and anthocyanin treated mice were fed 11g/ kg anthocyanin in place of 11g/ kg sucrose in the high fat diet only mice, which could well have contributed to the difference. Also, it was noted that food intake was lower in these mice compared to the control group.

5.1.8 Purpose of investigation

The chapter begins with identification of effects of lingonberry and cranberry extracts on FOXO1a phosphorylation. These extracts were fractionated using the experience that had been gained studying grape seed and pine bark extracts. The aim, as in chapter 3, was to learn more about the individual molecules or groups of molecules involved in mediating the cellular effects. By changing the extract under investigation it was hoped that this might become more tractable.

5.2 Results

Lingonberry (LB) and cranberry (CB) extracts samples were prepared as described in chapter 2. Solid phase extraction on Supelco SPE columns was used to fractionate the samples into unbound and bound fractions. Total phenol assays showed that the majority of phenolic compounds were recovered in the bound fractions (Table 5.2):

Sample	% of total recovery	Sample	% of total recovery
L UB1	5.5	C U1	7.1
L UB2	2.6		
L B1	63.8	C B1	92.1
L B2	11.0		

Table 5.2: Recovery of phenols in lingonberry and cranberry fractions after SPE
A total phenols assay was carried out to determine the phenolic content of each fraction.

L UB1- lingonberry unbound 1
L UB2- lingonberry unbound 2
L B1- lingonberry bound 1
L B2- lingonberry bound 2
CU1 – cranberry unbound 1
CB1 – cranberry bound 1

5.2.1 Comparison of the effectiveness of lingonberry and cranberry extracts in inducing FOXO1a phosphorylation

The lingonberry extract (LB) was more effective than the cranberry extract (CB) in inducing FOXO1 phosphorylation (Fig 5.5):

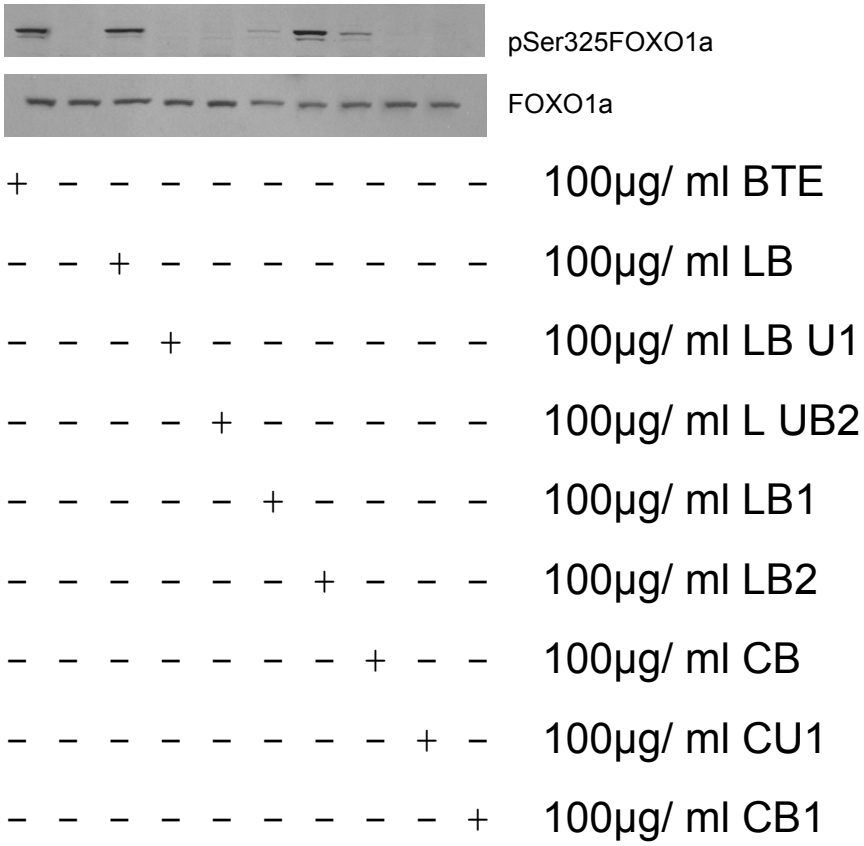


Fig 5.5: Effect of LB and CB and their fractions on FOXO1a phosphorylation
HEK 293 cells were cultured as described in the methods, followed by withdrawal of serum for 30 min. Cells were then stimulated for 30 minutes as above with 100µg/ml of the LB and CB whole extract or unbound/bound fractions. BTE was used as the positive control and vehicle only (DMEM) as the negative control. Cells were lysed and prepared for SDS-PAGE as described in the methods, followed by immunoblotting with an antibody that detects FOXO1a only when it is phosphorylated on the insulin-sensitive residue pSer 325 pFOXO1a) as well as a second antibody that detects FOXO1a regardless of phosphorylation state (FOXO1a).

After SPE fractionation, the activity was recovered in the second bound fraction of the LB whereas the LB unbound fractions showed no activity at this dose. This suggests that the most active components were recovered in this bound fraction. In the case of cranberry, although the original extract was effective, phosphorylation-stimulating activity was not recovered in the unbound or bound fractions. This suggests that either the active components were destroyed or did not elute from the SPE matrix under these conditions.

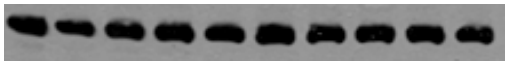
5.2.2 Anthocyanin content of SPE fractions

The SPE fractionation of CB was repeated with certain changes. Firstly, the SPE units were washed more thoroughly. Secondly, a vacuum pump was not used which ensured that the sample had longer to bind to the SPE matrix. As before, the majority of the phenolic material eluted in the bound fractions (Table 5.4). The fractions from this attempt were applied to cells and the blot repeated but using a higher concentration (200µg GAE/ml). It was apparent that the active components were recovered in the first bound sample and these were sufficient to induce phosphorylation of FOXO1a (Fig 5.6). At this higher dose, some activity was also seen in the later unbound washes.

Sample	% recovery total phenols
Unbound1	6.4
Unbound2	9.8
Unbound3	9.9
Unbound4	10.3
Unbound5	12.4
Unbound6	4.6
Bound1	15.5
Bound2	4.8

Table 5.3: The distribution of phenolic compounds after SPE of CB.

pSer325FOXO1a



FOXO1a

−	+	−	−	−	−	−	−	−	−	100µg/ ml BTE (positive control
−	−	+	−	−	−	−	−	−	−	200µg/ ml C U1
−	−	−	+	−	−	−	−	−	−	200µg/ ml C U2
−	−	−	−	+	−	−	−	−	−	200µg/ ml C U3
−	−	−	−	−	+	−	−	−	−	200µg/ ml C U4
−	−	−	−	−	−	+	−	−	−	200µg/ ml C U5
−	−	−	−	−	−	−	+	−	−	200µg/ ml C U6
−	−	−	−	−	−	−	−	+	−	200µg/ ml C B1
−	−	−	−	−	−	−	−	−	+	200µg/ ml C B2

Fig 5.6: Effect of CB fractions on FOXO1a phosphorylation

HEK 293 cells were cultured as described in the methods, followed by withdrawal of serum for 30 min. Cells were then stimulated for 30 minutes as above with 200µg/ml of the CB whole extract or unbound/bound fractions. 100µg/ ml BTE was used as the positive control and vehicle only (DMEM) as the negative control. Cells were lysed and prepared for SDS-PAGE as described in the methods, followed by immunoblotting with an antibody that detects FOXO1a only when it is phosphorylated on the insulin-sensitive residue pSer 325 pFOXO1a) as well as a second antibody that detects FOXO1a regardless of phosphorylation state (FOXO1a).

5.3 Identification of active components in the cranberry extracts using LCMS

LCMS analysis was used to characterise the polyphenol composition of the CB and LB extracts and their unbound and bound fractions. The composition of the cranberry extracts is summarised in Table 5.5 and Figure 5.7. The MS data is shown in Fig 5.8.

Peak no	Retention time (mins)	PDA max.	M/Z	MS^2	Putative ID
1	8.0	280	779	389	Unknown
2	8.5	300	575	341	PAC dimer (A)
3	9.1	280	ND	ND	Not identified
4	12.0	295	387	341, 179	Caffeoyl hexose
5	13.1	285	315	153	dihydroxy benzoic acid hexose
6	15.3	290	353	191	Chlorogenic acid
7	15.6	315	341	179	caffeoyl hexose
8	16.6	515, 285	447	285	Cyanidin hexose
9	17.4	315	341	179	caffeoyl hexose
10	17.7	515, 325, 285	353	191	Chlorogenic acid/ Anthocyanin
11	18.2	315	461	415, 293, 191	HCA derivative
12	19.2	275	329	283, 121	Benzoic acid hexose
13	20.4	275	513, 401, 289	401, 289	PAC derivative
14	20.8	280	863	711, 289	PAC trimer (A)
15	21.6	355	479, 317	317	Myricetin hexose
16	23.7	350	463, 301	301	Quercetin hexose
17	25.0	285	1151 575	ND ND	Proanthocyanidin tetramer (A) Proanthocyanidin dimer (A)
18	25.7	350	433, 301	301	Quercetin pentose
19	25.9	355	447	301	Quercetin rhamnose
20	29.1	350	317	ND	Myricetin
21	29.8	275	609 121	463, 301 No MS	Quercetin coumaroylhexose Benzoic acid
22	32.3	350	567, 301	301	Quercetin benzoyl hexose
23	33.8	350	310	ND	Quercetin

Table 5.4: Identification of phenolic compounds in cranberry extracts

Putative IDs for compounds in CB were collated from data in Antolovitch, 2004; McDougall, 2008; McDougall, 2010; Passos, 2007 and Weaver, 2009. PDA represents the maximal wavelength of absorption, *m/z* the mass to charge ratio and MS^2, the breakdown products generated with a collision factor of 45% normalised energy. The peak numbering refers to Fig. 5.7.

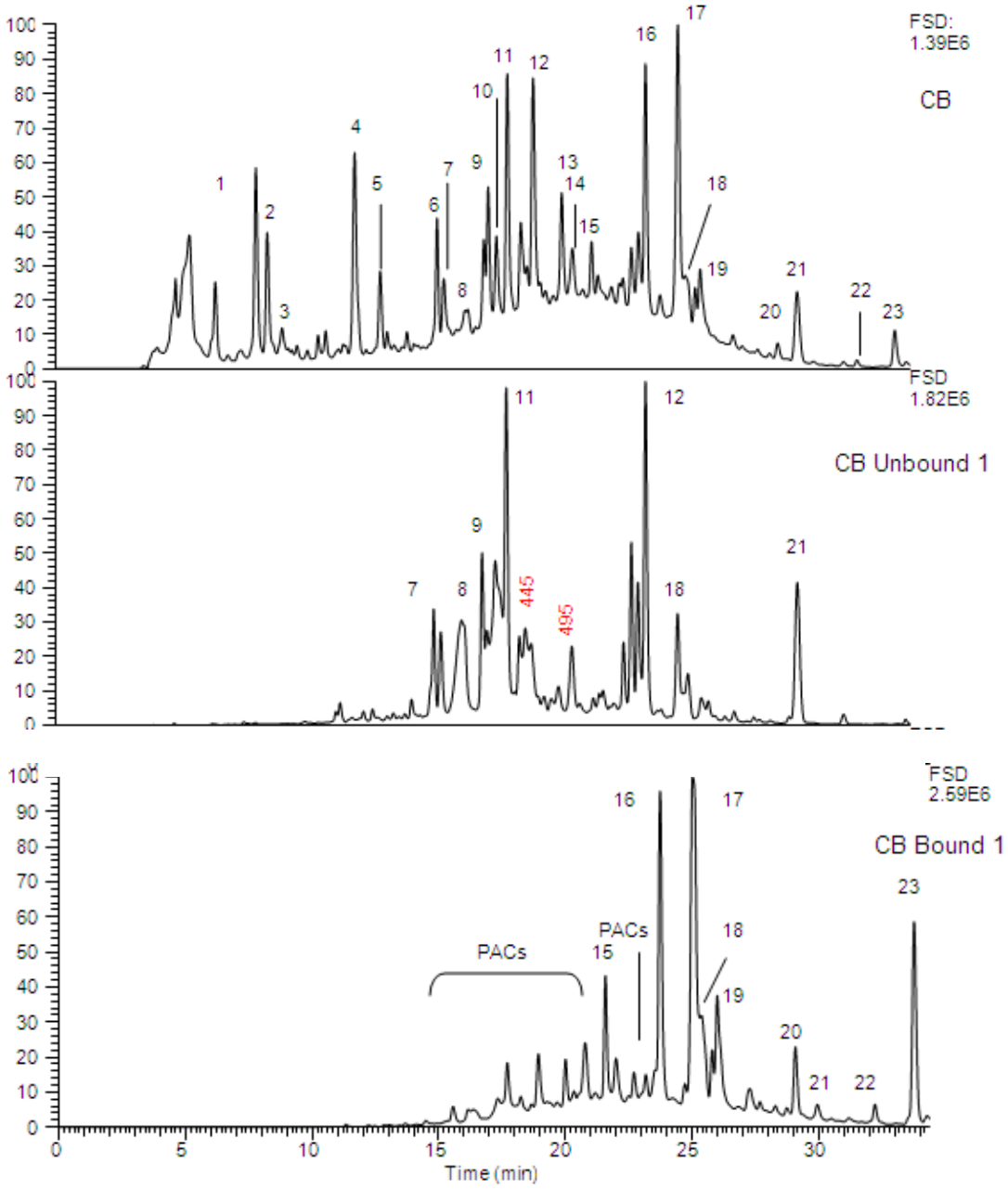


Fig 5.7: UV traces of CB fractions obtained by solid phase extraction

CB extract was fractionated by SPE as detailed in the methods. The figures in the top right corner denote the full scale deflection of the detector at 280 nm. Three fractions were subjected to LCMS as detailed in the methods. Some compounds were concentrated in the fractions that were too dilute to observe in the original sample. These are highlighted in red. PACs denotes an area rich in unresolved PACs. Numbering denotes peaks discussed in Table 5.5.

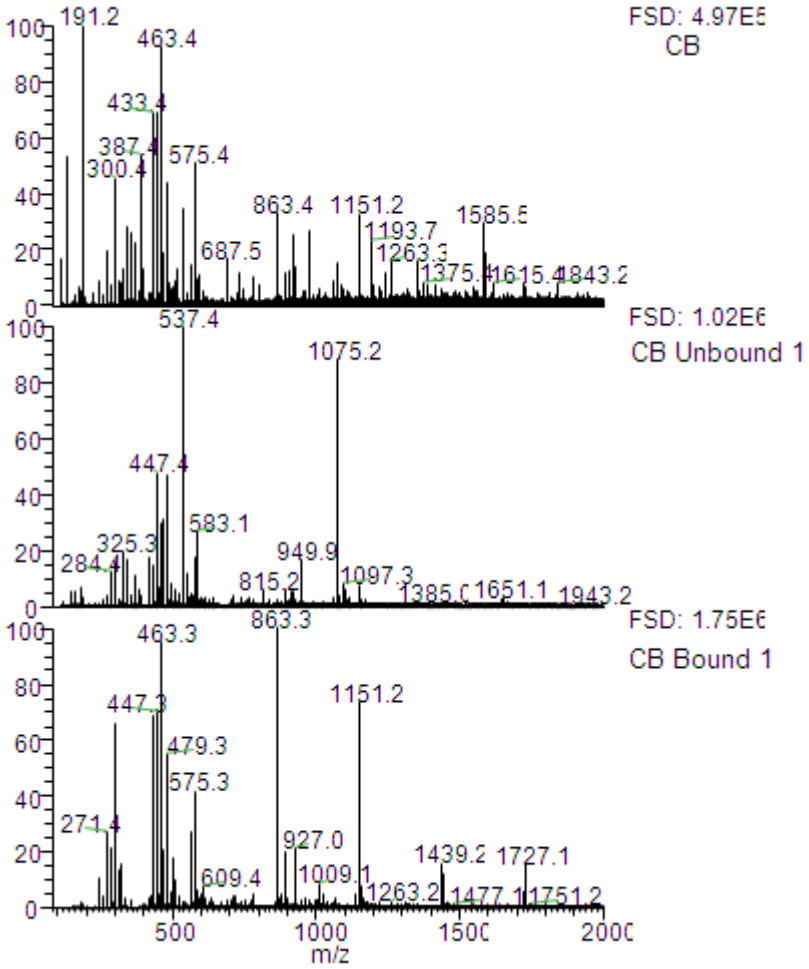


Fig 5.8: MS spectra of CB fractions obtained by solid phase extraction.
The figures in the top right corner denote the full scale deflection of the MS detector.

The original cranberry extract contained a mixture of polyphenol components, mainly hydroxycinnamic and hydroxybenzoic acid derivatives, flavonols, proanthocyanidins and some anthocyanins. The overall composition was similar to previous reports (Prior *et al*, 2001). Fig. 5.7 illustrates the fractionation of phenolic components on the Supelco columns. The components displayed differential affinity for the column matrix and the unbound sample was enriched in anthocyanins, hydroxycinnamic acid and hydroxybenzoic acid derivatives. In addition, certain components that were hidden in the original extract became apparent in the unbound sample, including some hydroxycinnamic acid derivatives that are characteristic of cranberry. For example, there are a cluster of peaks that were obscured by the abundance of peak 16 (quercetin hexose) in the original extract that have LC-MS properties that identify them as caviunin hexose derivatives (*m/z* 535 and 537; Iswaldi, 2012). Also, the presence of benzoic acid in peak 21 was more apparent in the unbound sample.

As outlined in the original method paper (Hellstrom, 2007), elution with aqueous acetone released the bound components and proanthocyanidins and flavonols were enriched in this fraction. The CB bound 1 fraction was enriched in some flavonols including peak 16 (quercetin hexose) and peaks 20 and 23 (myricetin and quercetin resp.). Certain PAC peaks were clearly eniched (peak 17; PAC tetramer). However, enrichment in PACs can be more readily displayed by comparing the MS spectra of the bound, unbound and original samples (Fig. 5.8) because these components do not separate well on RP-HPLC. It can be clearly noted that the MS spectra of the bound fraction is enriched in signals characteristic of a range of PACs and signals

from flavonols. It was also noted that the PAC concentrations in CB were low in comparison to other samples. It is possible that the PACs were removed when this commercially available juice was being processed.

5.4 Identification of active components in the lingonberry extracts using LC-MS

The LCMS data for lingonberry samples are shown in Table 5.6 and Figs 5.9 and 5.10.

Peak no	Retention time (mins)	PDA	M/ Z	MS_2	Putative ID
1	9.9	280	865.1	Multiple	PAC trimer (B)
2	14.0	280	577.0	407.1, 289.1	PAC dimer (B)
3	14.4	280, 515	447.0	285.1	Cyanidin hexose
4	15.1	280	577.0	425.0, 289.0	PAC dimer (B)
5	15.7	280	865.1	693.0, 577.1	PAC trimer (B)
6	16.0	280	865.1	695.1, 575.1	PAC trimer (B)
7	17.0	275	461.0	414.9	Not identified
8	18.1	275	863.0	711.0	PAC trimer (A)
9	18.6	280	865.1	Multiple	PAC trimer (B)
10	21.0	280	575.1	377.0, 449.1	PAC dimer (A)
11	21.1	280	355.1, 193.0	193.0	Feruloyl hexose
12	22.2	355	463.0	301.0	Quercetin hexose
13	22.7	285	861.1	700.0, 573.0	PAC derivative
14	24.3	350	433.0	323.0	Quercetin pentose
15	24.6	265, 350	447.0	301.0	Quercetin rhamnose
16	27.8	350	591.1	447.0, 489.1	Quercetin-HMG rhamnose
17	28.5	275	121.1	ND	Benzoic acid
18	29.1	290, 325	193.1	193.1, 161.0	Ferulic acid
19	30.2	350	575.1, 285.2	445, 285	Kaempferol-HMG rhamnose
20	30.9	345	567.1, 301.1	447.1, 301.1	Quercetin benzoyl hexose
21	31.7	345	567.1, 301.1	447.1, 301.1	Quercetin benzoyl hexose
22	32.1	365	301.1	165.0	Quercetin

Table 5.5: Identification of phenolic compounds in lingonberry extracts

Putative IDs for compounds in LB were collated from data in Antolovitch, 2004; McDougall, 2008; McDougall, 2010;Passos, 2007 and Weaver, 2009. PDA represents the maximal wavelength of absorption, *m/z* the mass to charge ratio and MS^2, the breakdown products generated with a collision factor of 45% normalised energy. The peak numbering refers to Fig. 5.9.

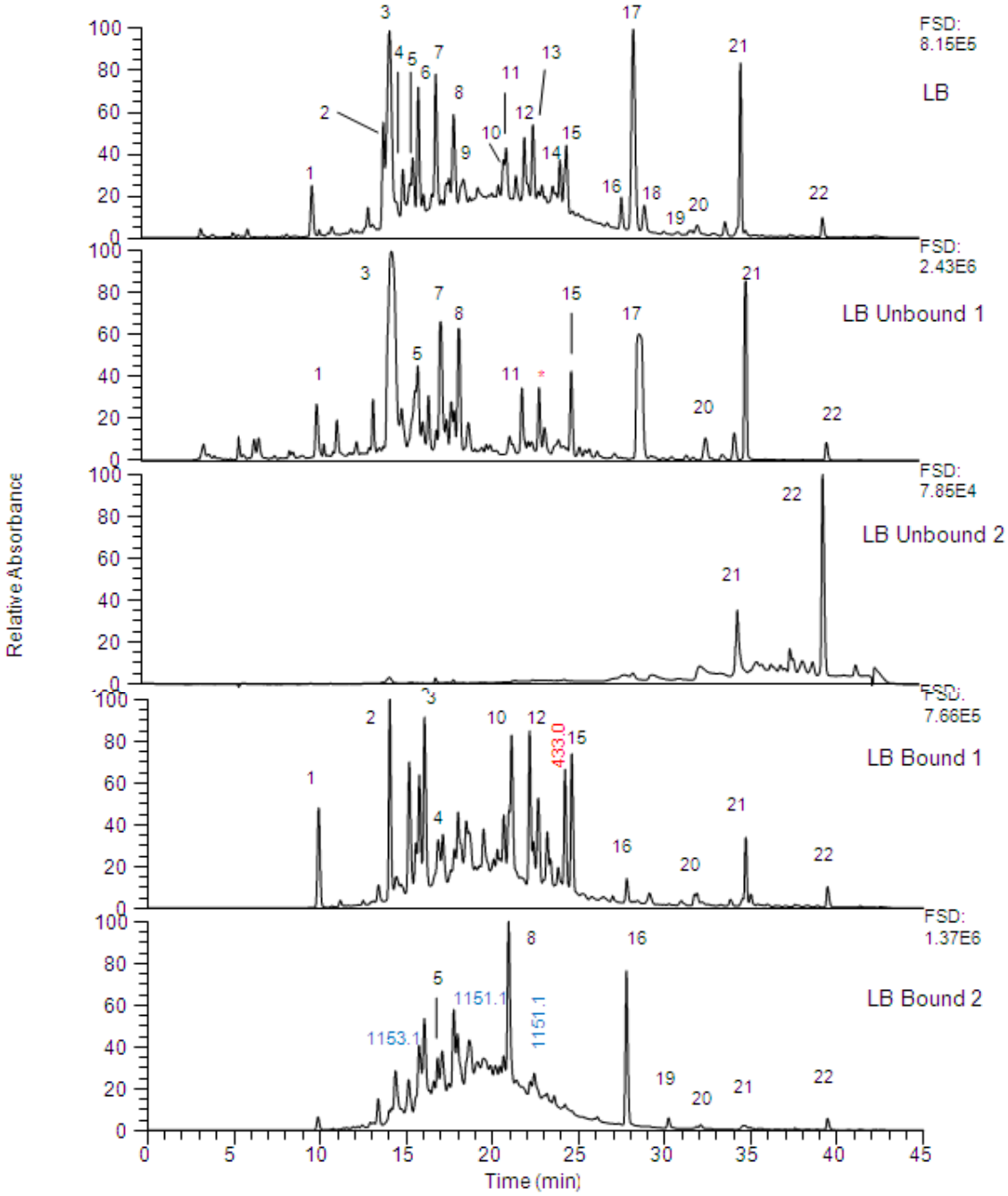


Fig 5.9: Chromatograms of LB fractionated by solid phase extraction

LB extract was fractionated by SPE as detailed in the methods. Five fractions were subjected to LCMS as detailed in the methods. The figures in the top right corner denote the full scale deflection of the detector at 280 nm. Some more concentrated compounds were observed in the fractions produced that were too dilute to observe in the original sample. These m/z values are highlighted in red. Numbering follows format discussed in Table 5.6.

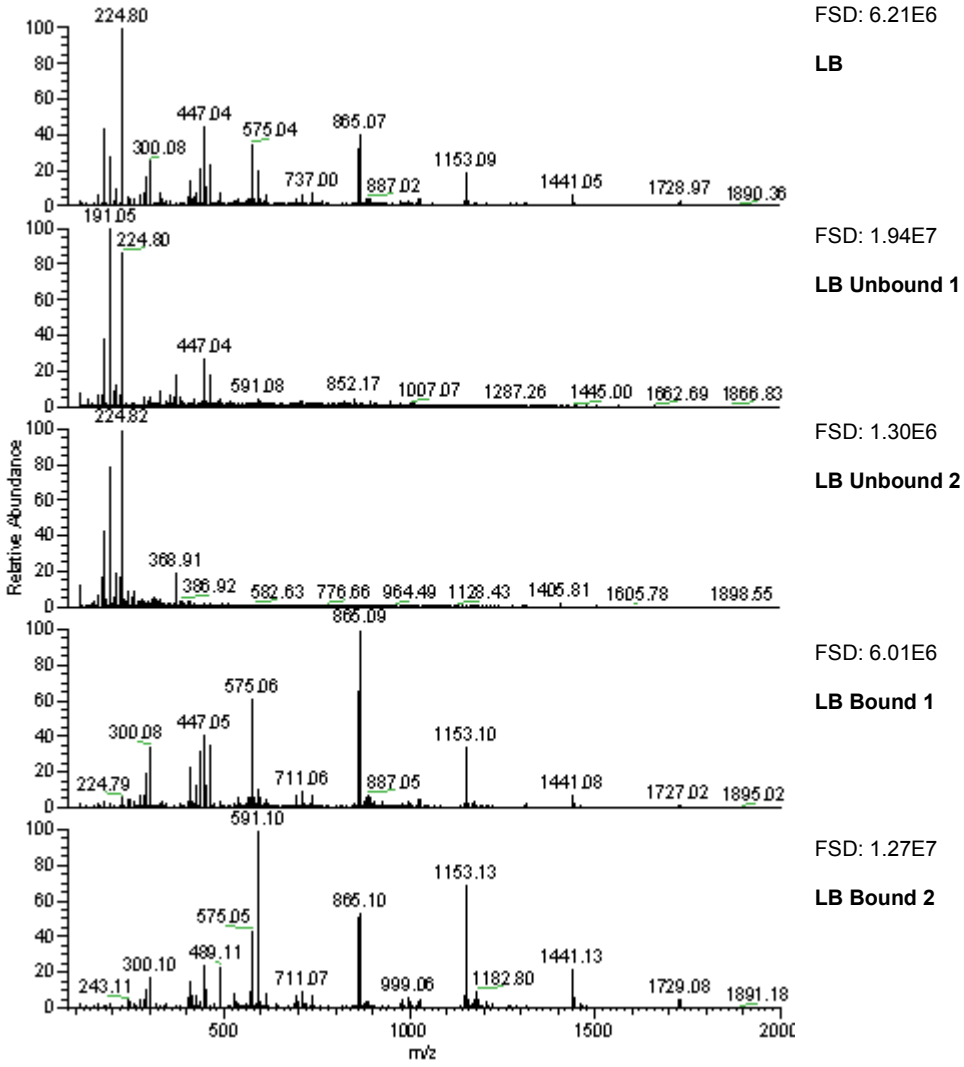


Fig 5.10: MS of LB obtained by solid phase extraction
Fractions were subjected to mass spectrometry as detailed in the m
the top right corner denote the full scale deflection of the MS detector.

The polyphenol composition of the lingonberry extract matched previous reports (Prior *et al*, 2001; Ek *et al*, 2006; McDougall *et al*, 2008A) and contained a diverse mixture of anthocyanins, hydroxycinnamic acid derivatives, flavonols and proanthocyanidins. It also contained substantial amounts of both ferulic acid and benzoic acid, which is characteristic of lingonberry (McDougall *et al*, 2008B; Visti *et al*, 2003). After fractionation on SPE, the unbound sample was enriched in anthocyanins, hydroxycinnamic acid derivatives, hydroxybenzoic acid derivatives and benzoic acid. Some peaks become more apparent in the unbound sample following the removal of other peaks, e.g. a second feruloyl hexose appears (see asterisk, Fig 5. 9). Once again, the bound fractions were relatively enriched in certain flavonols and PACs. For example, peak 16 was identified as quercetin hydroxymethylglutaroyl (HMG) rhamnose and peaks 5 and 8 as PAC trimers. The enrichment in PACs in LB bound 2 was also apparent in the hump of unresolved peaks between 8-25 mins. The enrichment in PACs and flavonols was mirrored in the MS spectra (Fig. 5.10).

5.5 Comparison of the spectra of active fractions

Cell-based studies had also shown that the most active LB bound fraction was substantially more effective than the most active CB bound fraction. Comparing these PAC-rich fractions further, it is clear that the most-active LB bound fraction (LB bound 2) contained a higher proportion of predominately B-type PACs than the less active CB bound 1 fraction (see Figs. 5.8 and

5.10). In addition, the LB bound 2 fraction appeared to contain a larger proportion of higher molecular weight PACs.

The data also suggested that LB bound fraction 2 was considerably more effective than LB bound 1 in inducing phosphorylation of FOXO1a (Fig 5.5). The LC-MS runs and MS spectra data (Figs 5.9 and 5.10) show that both bound fractions were enriched in PACs and certain flavonols. The bound fraction 1 contained a greater proportion of smaller PACs (trimers and below) and probably contained a greater proportion of flavonols. The bound fraction 2 was more enriched in PACs as it only contained substantial amounts of one flavonol (peak 16, quercetin-hydroxymethylglutaroyl rhamnose). Also, the PACs present appeared to be of generally higher DP (trimers and above). Overall, this suggests that PACs may be more effective than flavonols in inducing phosphorylation of FOXO1a and that PACs of higher molecular weight may also be more effective.

5.6 Dose-response effect of the most active LB fraction on FOXO1a phosphorylation

Dose response experiments were carried out to assess how effective the LB bound 2 was at lower concentrations. Phosphorylation of FOXO1a was apparent at 25 µg /ml (Fig 5.11) and did not increase greatly at 100 µg/ml of LB bound 2 fraction.

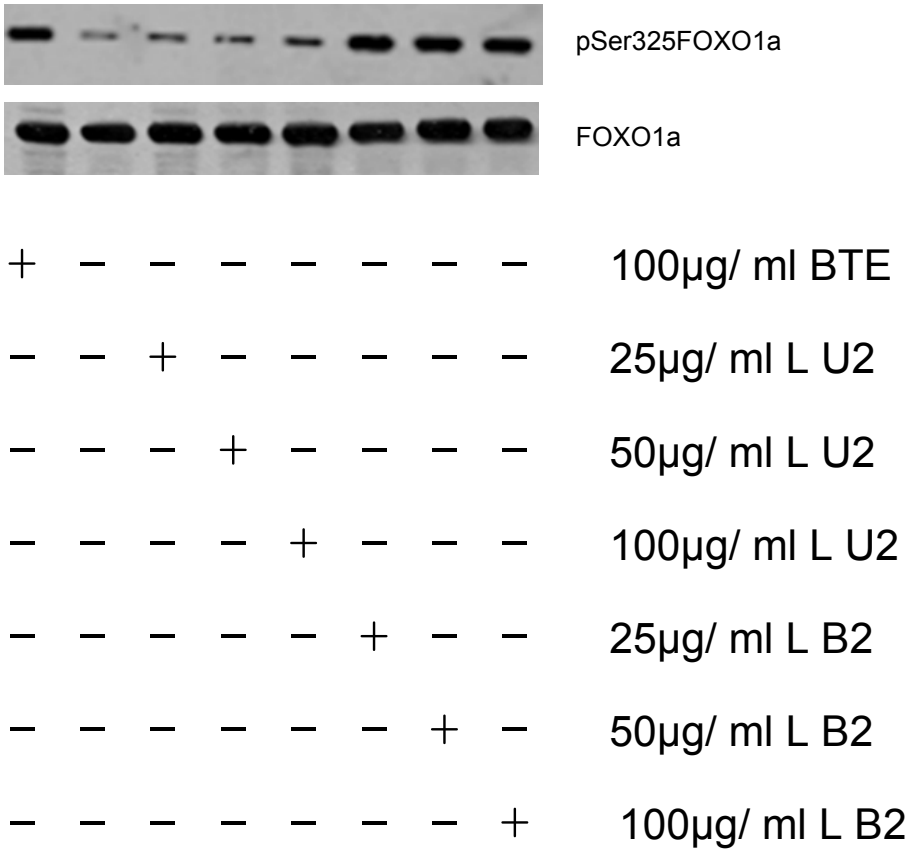


Fig 5.11: Effect of different doses of LB bound 2 fraction on FOXO1a phosphorylation
HEK 293 cells were cultured as described in the methods, followed by withdrawal of serum for 30 min. Cells were then stimulated for 30 minutes as above with 100µg/ml of the LB and CB whole extract or unbound/bound fractions. 100µg/ ml BTE was used as the positive control. Cells were lysed and prepared for SDS-PAGE as described in the methods, followed by immunoblotting with an antibody that detects FOXO1a only when it is phosphorylated on the insulin-sensitive residue pSer 325 pFOXO1a) as well as a second antibody that detects FOXO1a regardless of phosphorylation state (FOXO1a).

5.7 Fate of lingonberry components during incubation with cells

HEK293 cells were treated with 100 µg/ml LB for 0, 15, 30, 45, 60 and 75 mins and the media subjected to LCMS to track the fate of the phenolic components. Over time, the larger PACs disappeared from the LCMS trace of the media beginning at between 15 mins and were completely absent by 60mins. There was a suggestion that flavonol components (e.g. peak I and

peak D) survived for longer but this could not be determined with confidence. This was mainly because the media contained components that interfered with detection of peaks (e.g. the resilience of peak 4 is mainly due to the presence of the pH indicator dye in the media) or ions with similar *m/z* that overwhelmed the phenolic components.

The larger PACs may have bound to other components of the media (Porter, 1993). It is also possible that the larger PACs were being transported into the cell or were bound to or sequestered into the plasma membrane perhaps due to their lipophilic nature or simply that degradation occurred in the extracellular medium. Further work could examine for the uptake of these components or the presence of their metabolites inside the cells. This data is shown in Fig 5.12 and 5.13 and table 5.6:

Peak	m/z	ID	0	15	30	45	60
A	865.1	PAC trimer (B)	865.1				
B	863.0	PAC trimer (A)	863.0				
C	865.1	PAC trimer (B)	865.1	865.1	865.1		
D	463.0	Quercetin hexose	463.0	463.0	463.0	463.0	
E	433.0 575.1 863.0	Quercetin pentose PAC dimer (A) PAC trimer (A)	433.0 575.1 863.0	433.0 575.1 863.0	433.0 575.1 863.0	 863.0	
F	433.0 575.1 865.1 1153.1	Quercetin pentose PAC dimer (A) PAC trimer (B) PAC tetramer (B)	433.0 575.1 865.1 1153.1	433.0 575.1 1153.1	433.0 575.1	 575.1	
G	575.1 863.0 1153.1	PAC dimer (A) PAC trimer (A) PAC tetramer (A)	575.1 863.0 1153.1				
H	575.1 863.1 1151.1	PAC dimer (A) PAC trimer (A) PAC tetramer (B)	575.1 863.1 1151.1				
I	591.1	Quercetin HMG rhamnose	591.1	591.1	591.1	591.1	

Table 5.6 Time course study of PAC presence in DMEM. HEK293 cells were treated with 100 µg/ml LB for 0, 15, 30, 45, 60 and 75 mins and the media subjected to LCMS at 45^{o}C. The presence of characteristic MS signals was assessed over 60 mins.

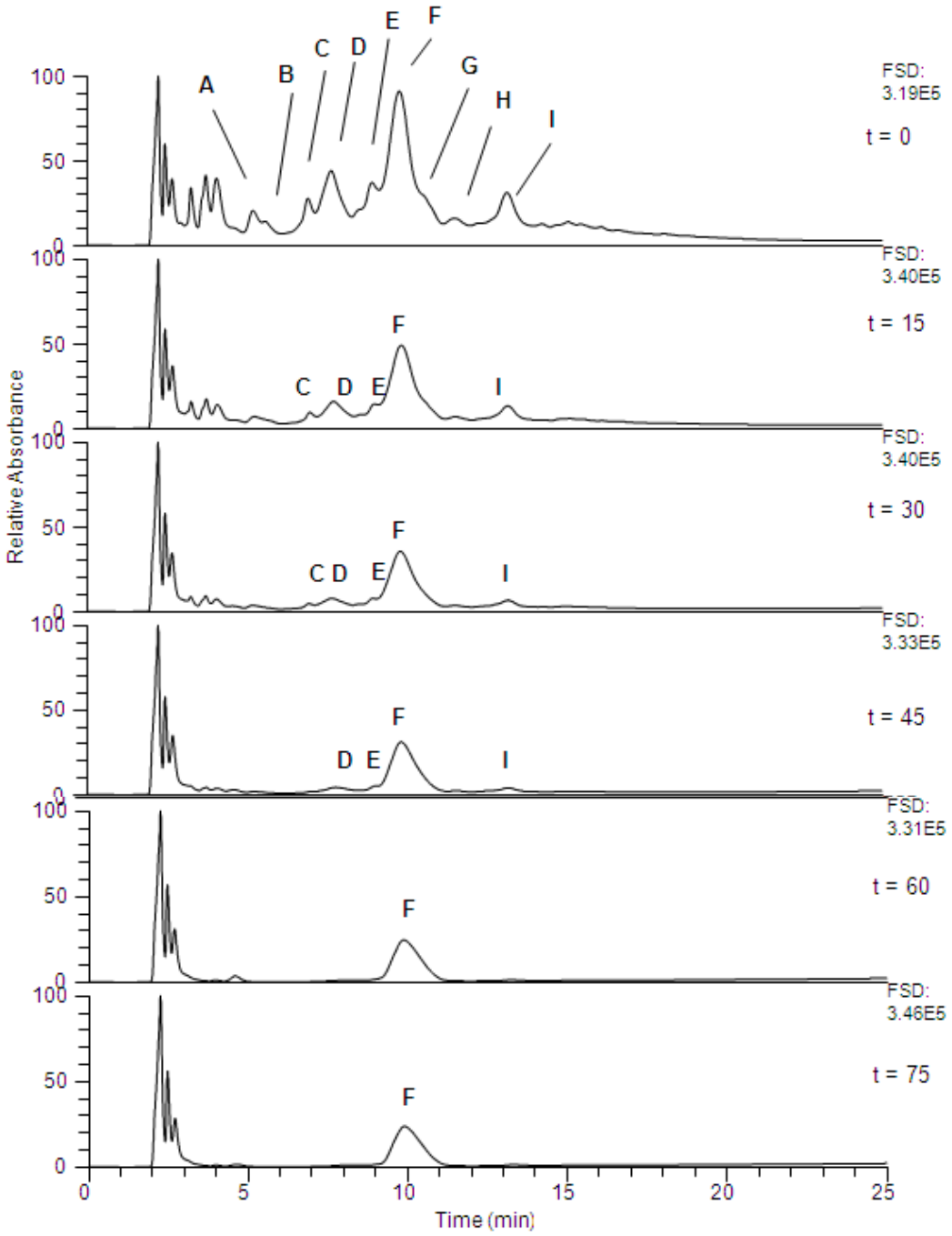


Fig 5.12: Comparison of chromatograms of LB exposed to cells and DMEM over a time course.
HEK293 cells were placed in serum free medium and LB added. At the different time points shown, DMEM was taken for analysis by LCMS. The figures in the top right corners represent the full scale deflection of the detector at 280 nm. Peaks A-I are from the LB extract and are discussed in the text and in Table 5.6.

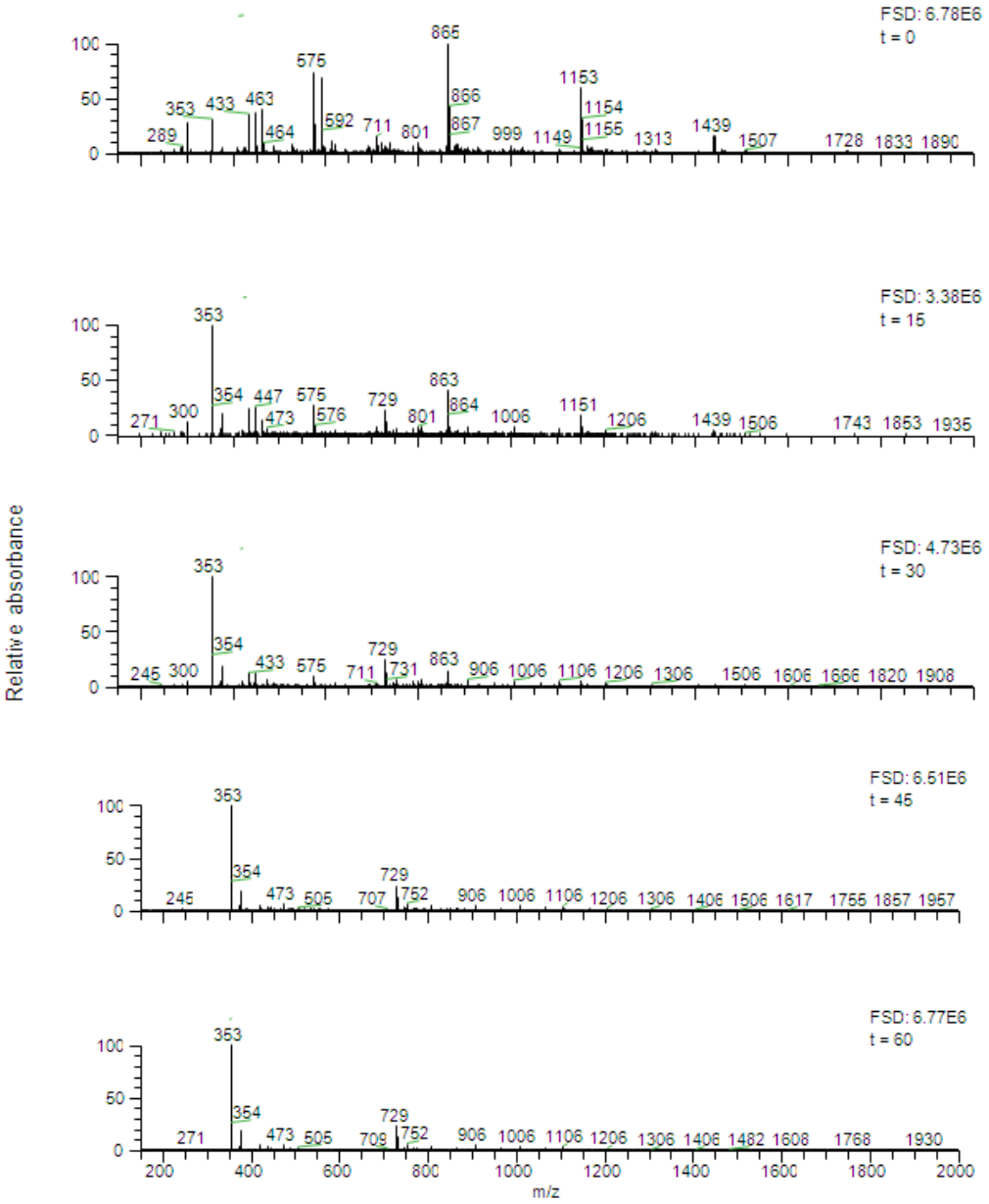


Fig 5.13: MS data from LB media recovery experiment.
HEK293 cells were placed in serum free medium and LB added. At the different time points shown, DMEM was taken for analysis by LCMS. T=75mins not included because it was identical to t=60mins. The figures in the top right corners represent the full scale deflection.

Discussion

5.8 LB is more effective than CB in inducing FOXO1a phosphorylation

Cell culture and western blot analysis confirmed that LB was more effective at inducing phosphorylation of FOXO1a than CB. The induction of FOXO1a phosphoylation by phenolic rich extracts of CB and LB confirm previous results obtained with black tea theaflavins (Cameron *et al*, 2008). It proved impossible to identify individual structures mediating the cellular effects of LB and CB and therefore no further characterisation of such agents could be carried out. Despite this, LCMS analysis of the extracts suggested that the most effective fractions contained:

1. a higher level of PACs
2. the presence of larger PACs in active compared to inactive fractions
3. more B type PACs in LB compared to CB

5.9 Role of linkage type and degree of polymerisation in effects of PACs on other model systems

LCMS analysis suggested that the most active fraction of LB contained a higher proportion of larger molecules than in the inactive samples. Other studies also suggested that the levels of the PACs began to decline in the extracellular media within 15 minutes of treatment and they were undetectable by 60 minutes. This may be due to the breakdown of PACs to monomers which can be predicted to be taken up into the cell and thereby exert their effects on FOXO1a. Indeed, PAC monomers and dimers are known to be taken up by epithelial cells (Deprez *et al*, 2001). Initial work to identify which phenolic compounds entered the cells was unsuccessful,

probably because the levels were below detection levels of the MS techniques employed. Further work using labelled compounds may be required to determine if monomers or indeed PACs can be detected within the cells

It was noted that LB, which was more effective with respect to inducing phosphorylation of FOXO1a, contained more B type PACs than CB, which contained predominately A type PACs. and in view of the results presented here, further study into the differential effects of A type versus B type may be of interest.

5.10 Effects of flavonols compared to PACs

The data presented here shows that samples more enriched in PACs than flavonols were more effective at inducing phosphorylation of FOXO1a than those with more substantial levels of flavonols. This suggests PACs may be more effective than flavonols.

In conclusion, this chapter investigated the ability of lingonberry and cranberry extracts to regulate the phosphorylation of the transcription factor FOXO1a. As in chapter 3, it was not possible to identify individual active structures for detailed characterisation. The analysis of the composition of active fractions that has been carried out may facilitate this in the future.

CHAPTER 6- BIGUANIDES AND DIGUANIDES

6.1 Introduction

Guanidines were originally extracted from French lilac (*G. officinalis)* (Witters, 2001) and were found to have blood glucose lowering effects in rabbits. While guanidine itself proved too toxic for clinical use, for example in one study 12 out of 21 rabbits died (Watanabe, 1918), galegine, synthalin and biguanides proved less toxic (Bailey and Day, 2004). Several of these agents have been used clinically since but only one, the biguanide metformin, remains in clinical use today (Witters, 2001). The basic guanidine structure is shown in Fig 6.1:

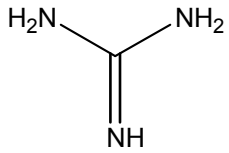


Fig 6.1: The basic guanidine structure.
Various modified forms of this structure are found in French lilac.

6.1.1 Galegine

Galegine (isoamylene guanidine) was briefly used as an antidiabetic agent in the 1920s. This molecule is illustrated in Fig 6.2:

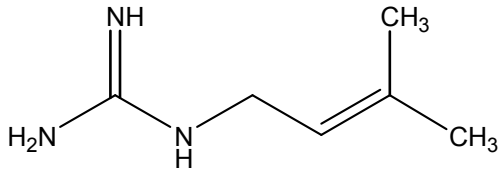


Fig 6.2: Structure of galegine

6.1.2 Biguanides

Biguanides are synthesised from 2 moles of guanidine with the elimination of one mole of ammonia (Kurzer and Pitchfork 1968) as shown in Fig 6.3:

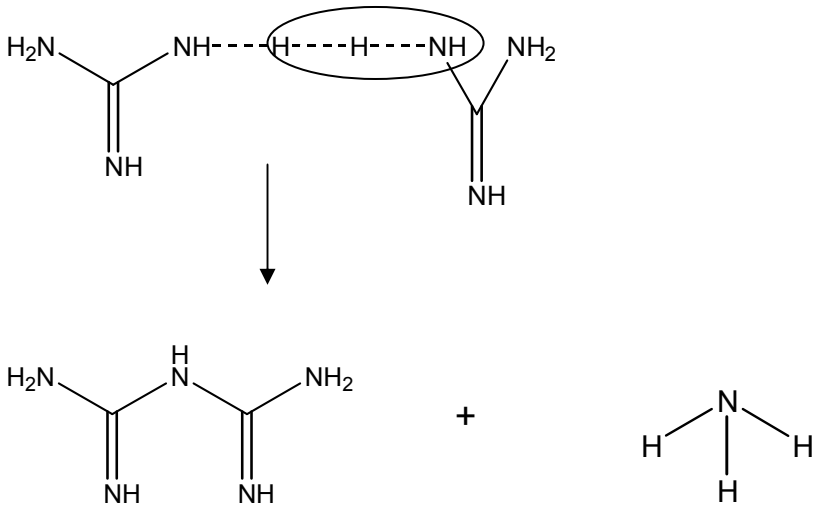


Fig 6.3: Synthesis of biguanide

6.1.2.1 Buformin

Buformin was used as an antihyperglycemic agent in the 1960s but was withdrawn in the 1970s due to an unacceptably high number of incidents of lactic acidosis and cardiac mortality (Witters, 2001). Lactic acidosis is a metabolic condition characterised by low tissue pH caused by elevated lactate levels. Lactate is a substrate for hepatic gluconeogenesis but metabolism of lactate in this way is inhibited by biguanides, probably due to inhibition of electron transport (Owen *et al*, 2000; El-Mir *et al,* 2000; Foretz *et al*, 2010), which will also promote anaerobic respiration, and accelerate lactate formation. The buformin structure is shown in Fig 6.4:

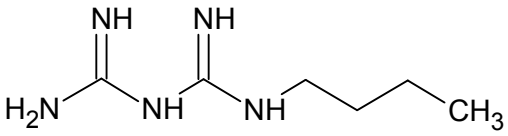


Fig 6.4: Structure of Buformin (butylbiguanide)

6.1.2.2 Phenformin

Phenformin started to be used as an antihyperglycemic agent in the 1950s (McKendry *et al,* 1959) but was withdrawn in the 1970s due to a high risk of lactic acidosis (Bailey, 1992) which was later associated with older age (Fimognari *et al*, 2008). The structure of phenformin is shown in Fig 6.5:

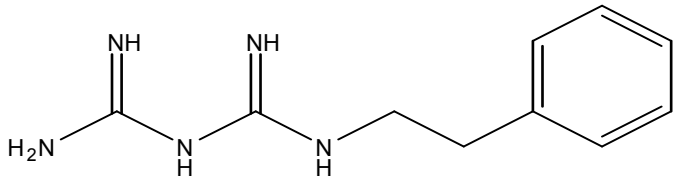


Fig 6.5: Structure of Phenformin (phenethylbiguanide).

6.1.2.3 Metformin

Metformin has been in use since 1957 and is only contraindicated in patients with renal failure as it is eliminated by the kidney (Bailey, 1992). Unlike phenformin, metformin does not induce lactic acidosis, probably because it is a less potent inhibitor of electron transport (Owen *et al*, 2000). The metformin structure is shown in Fig 6.6:

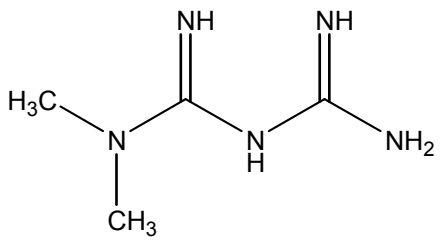


Fig 6.6: Structure of Metformin (dimethylbiguanide)

6.1.3 Diguanides

Diguanides are 2 guanidine molecules joined together by a hydrocarbon chain of variable length. The general diguanide structure is shown in Fig 6.7

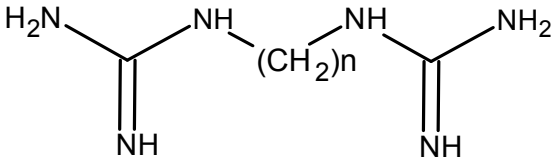


Fig 6.7: The general structure of diguanides**.**
The diguanide structure consists of 2 guanidine molecules bound together by a hydrocarbon chain of variable length.

6.1.4 Diguanides and related compounds that are commercially available

At the time of these experiments, there were three diguanides commercially available. Diguanide 4 (DG4) has been proposed as an N-Methyl-D-aspartic acid (NMDA) antagonist (Daeffler *et al*, 1999). It is illustrated in Fig 6.8:

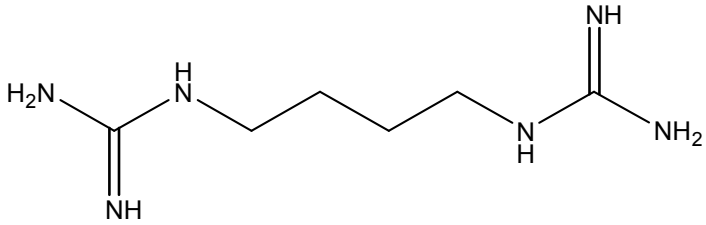


Fig 6.8: Structure of DG4 (arcaine)

The structures of other diguanides, DG6 and DG10 are shown in Fig 6.9 and 6.10:

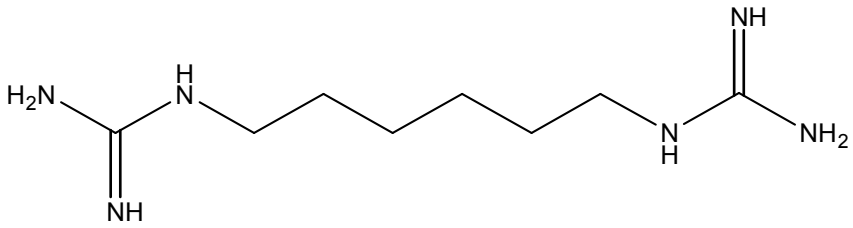


Fig 6.9: Structure of DG6 (hexamethylene diguanide)

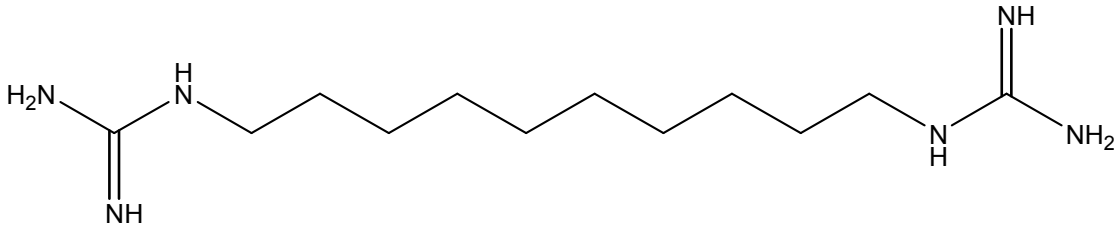


Fig 6.10: Structure of Synthalin A (DG10; decamethylene diguanide).

In the 1920s, before the discovery of the antihyperglycaemic properties of biguanides, these diguanides were briefly evaluated as antidiabetic agents. The clinical goals of treatment at the time, very different from those prevailing today, were to induce insulin-like 'hypoglycaemic' effects, as at this time the notions of type 1 and insulin-resistant type 2 diabetes were yet to be developed. In this series, the hypoglycaemic effects were found to increase, in proportion with toxicity, up to a linker size of 10-12 carbons (Bischoff *et al*, 1929). To achieve the maximum hypoglycaemic effect, the most potent and toxic diguanide, (*N*,*N*'-1,10-Decanediyl*bis*guanidine, synthalin A, termed DG10 in this chapter, fig 6.10) was used in humans, before the magnitude of its toxicity was fully realised and clinical use ceased. Synthalin B, fig 6.11, which is not available commercially, was considered to be less toxic but less

effective than synthalin A (Braun and Ludwig, 1937) and continued to be used in Germany until the 1940s (Bailey and Day, 2004).

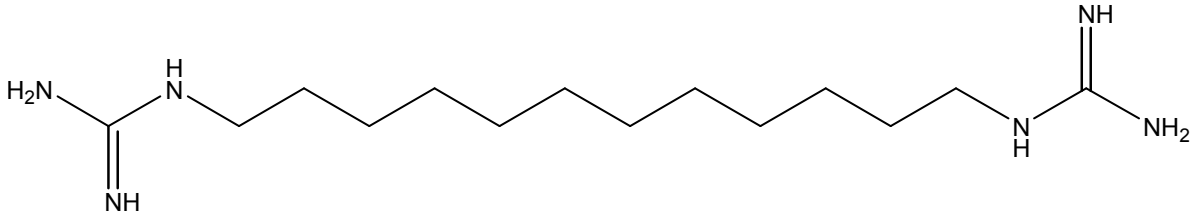


Fig 6.11: Structure of Synthalin B (DG12; neosynthalin; dodecylmethylene diguanide).

The final commercially available compound studied is an alkylguanide (Fig. 6.12). These are structurally similar to diguanides, except that they contain only one guanide group.

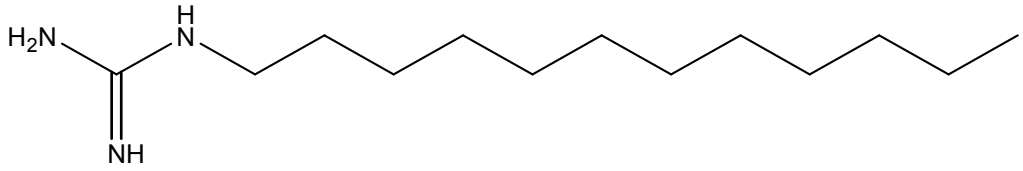


Fig 6.12: Structure of dodecylalkylguanide (AG12; dodin).

6.1.5 Comparison of biguanides and diguanides

Although metformin (dimethyl biguanide) is still in clinical use and considered to be effective and nontoxic, its use is problematic for some people. It can cause gastrointestinal problems, which leads to cessation of use in 5-10% of patients (Garber *et al*, 1997). In those that do take the drug, resistance occurs after a few years (Turner *et al*, 1999). If its mechanism of action could be confirmed, it may be possible to modify the structure in such a way as to avoid these responses in people vulnerable to them, whilst retaining their clinical efficacy. Comparative studies are one potential avenue of investigation with respect to this aim. Metformin is most typically compared with phenformin and sometimes buformin but in this chapter we have

compared metformin with diguanides. As a result of their early clinical failure, there has been little investigation of the diguanides in recent times but meanwhile understanding of the antihyperglycaemic action of metformin has greatly improved. Although the mechanism of antihyperglycaemic effects is incompletely understood for either drug class, inhibition of mitochondrial respiration has been observed in response to both diguanides (Pressman, 1963) and biguanides (Owen *et al*, 2000). In addition, in the last decade, and as discussed in the Introduction, it has been found that metformin activates AMPK (Zhou *et al*, 2001); however, the role of AMPK in antihyperglycaemic responses to these drugs has been reassessed in more recent studies. For example, studies on genetic models indicate that the clinically important effect of metformin on hepatic glucose output was AMPK-independent (Foretz *et al*, 2010) and in addition, regulation of S6 by metformin, which was thought to be AMPK-dependent, does not require AMPK either (Kickstein *et al*, 2010; Kalendar *et al*, 2010). It is currently unclear whether or not these negative data are indicative of redundancy in regulatory mechanisms, or alternatively that AMPK acts simply as a biomarker of antihyperglycaemic drug administration (Miller and Birnbaum, 2010). Whichever is the case, the graduated responses to diguanides provide an excellent additional opportunity to study antihyperglycaemic drug action because cellular responses mediating (or gauging) the antihyperglycaemic effects of diguanides will be expected to occur at much lower concentrations in diguanides possessing longer linkers than in those with shorter linkers. In the current chapter therefore, we have investigated the impact of linker length on gluconeogenic gene regulation and especially AMPK and S6 regulation. We

started by studying the commercially available compounds described above, before synthesising, assessing the purity and then characterising the effects of a series of other diguanide compounds on these cellular outputs.

6.2 Results

6.2.1 A DG carbon chain length of 6 or more is sufficient for maximal response

Firstly, the effects of commercially available diguanides on AMPK, ACC and S6 phosphorylation were investigated. H4IIE cells were treated with 25μM concentrations of each diguanide available and western blotting was carried out to detect phosphorylated ACC and AMPK, and dephosphorylated S6. This suggested a chain length of 6 carbons was sufficient to obtain maximal physiological effect. A fourth compound dodecylalkylguanidine (AG12) was also tested. AG12 is identical to DG12 except that it only contains one guanidine group (Fig. 6.12). This drug gave effects that were similar to those of DG10. These effects are similar to those achieved with metformin at millimolar, not micromolar concentrations. The data acquired is shown in Fig 6.13:

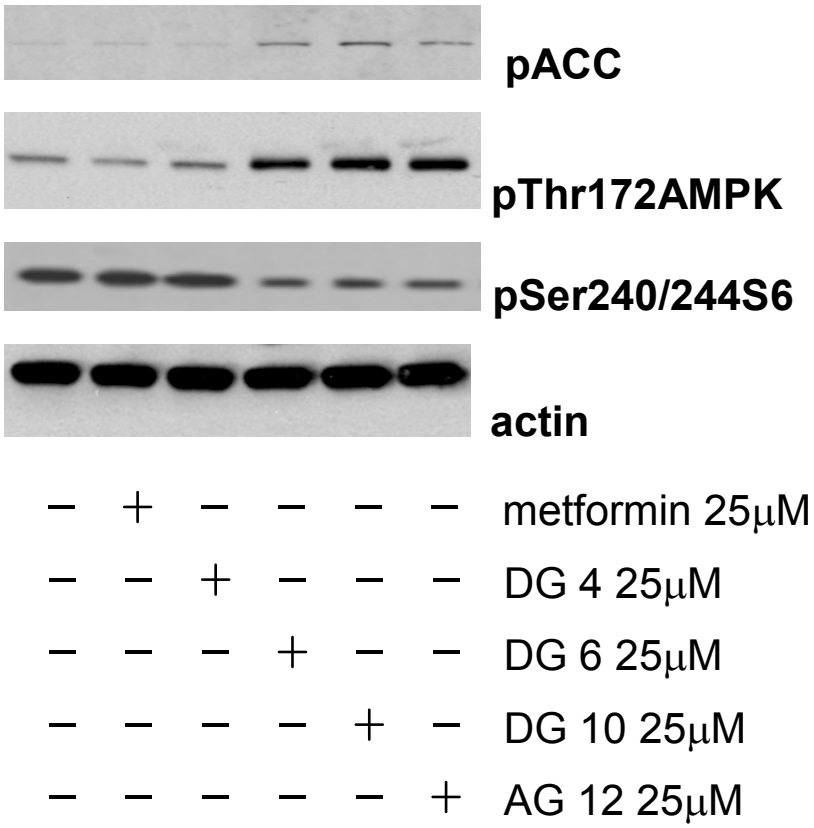


Fig 6.13: Effect of diguanides on cell signalling in H4IIE cells
Serum starved H4IIE cells were treated as above for 3hrs followed by lysis and SDS- PAGE as described in the chapter 2. Immunoblotting was carried out using two ACC antibodies- one which detects total ACC (ACC) and one which detects phosphorylated ACC (pACC), an antibody which detects AMPK only if it is phosphorylated on Thr172 (pThr172 AMPK), two S6 antibodies- one which detects total S6 (S6) and one which detects Ser240/244 S6 phosphorylation (pSer240/244 S6) and an actin antibody.

6.2.2 Effect of a dose response of DG6 on AMPK, S6 and ACC phosphorylation.

Cell culture and western blotting found that DG6 induced AMPK and ACC phosphorylation maximally and inhibited S6 phosphorylation almost maximally at 3.14μM. This is shown in Fig 6.14:

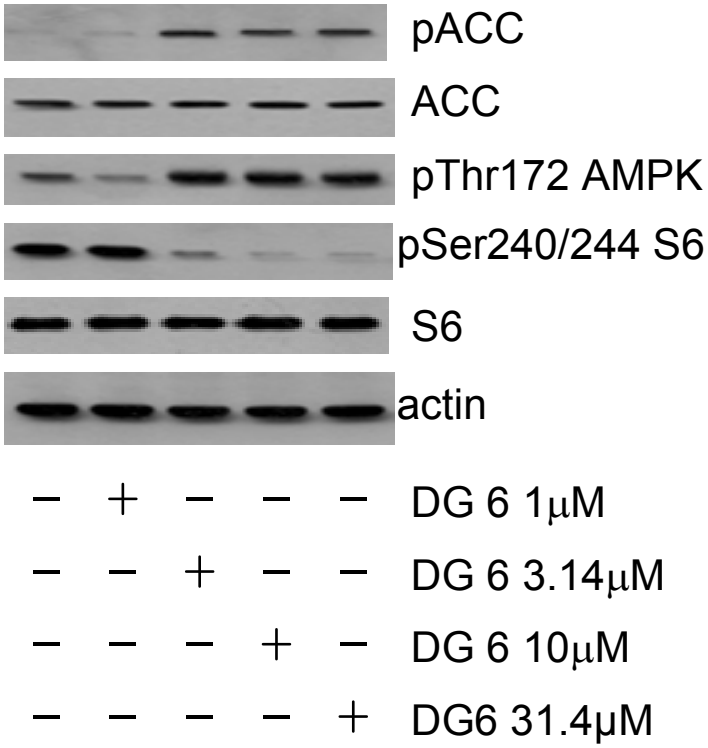


Fig 6.14: Effect of DG6 on AMPK, ACC and S6 phosphorylation.
Serum starved H4IIE cells were treated as above for 3hrs followed by lysis and SDS- PAGE as described in chapter 2. Immunoblotting was carried out using two ACC antibodies- one which detects total ACC (ACC) and one which detects phosphorylated ACC (pACC), an antibody which detects AMPK only if it is phosphorylated on Thr172 (pThr172 AMPK), two S6 antibodies- one which detects total S6 (S6) and one which detects Ser240/244 S6 phosphorylation (pSer240/244 S6) and an actin antibody.

6.2.3 Effect of a dose response of DG10 on AMPK, S6 and ACC phosphorylation.

Cell culture and western blotting found that DG10 (synthalin A) induced AMPK and ACC phosphorylation maximally and inhibited S6 phosphorylation at 3.14μM. This data is shown in Fig 6.15:

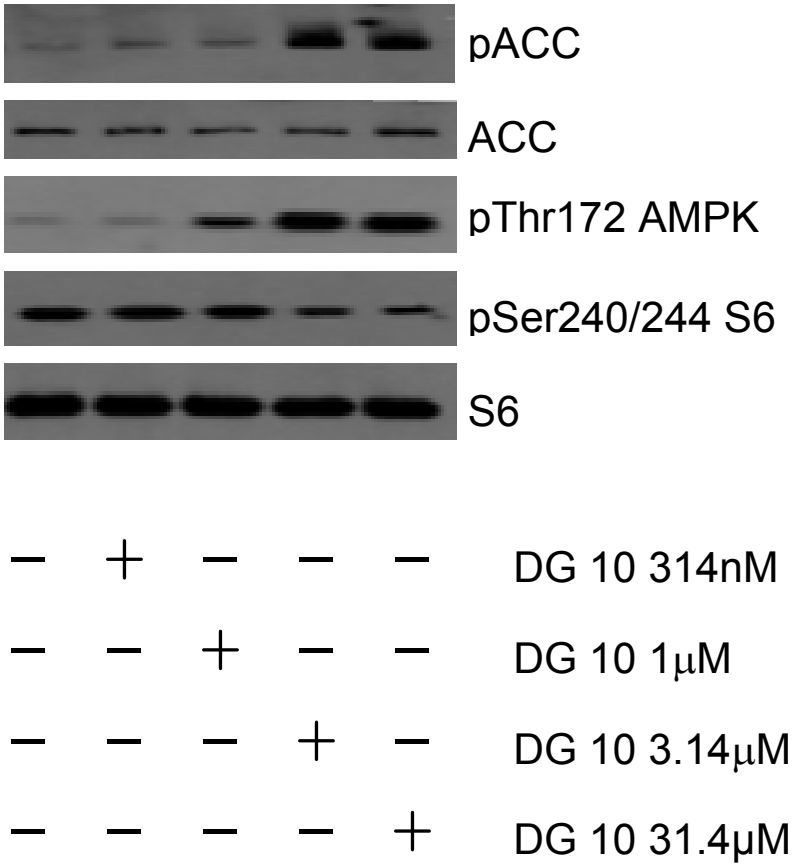


Fig 6.15: Effect of DG10 on AMPK, ACC & S6 phosphorylation
Serum starved H4IIE cells were treated as above for 3hrs followed by lysis and SDS- PAGE as described in chapter 2. Immunoblotting was carried out using 2 ACC antibodies- one which detects total ACC (ACC) and one which detects phosphorylated ACC (pACC), an antibody which detects AMPK only if it is phosphorylated on Thr172 (pThr172 AMPK), 2 S6 antibodies- one which detects total S6 (S6) and one which detects Ser240/244 S6 phosphorylation (pSer240/244 S6).

Taken together, the results so far show that there is a threshold in efficacy in this drug class, as DG4 was unable to regulate the signalling pathways under investigation, whilst both DG6 & DG10 exhibited efficacy in the low micromolar range. To further substantiate and investigate the threshold, more diguanides in the series were synthesised in the laboratory.

6.2.4 Diguanide synthesis

Diguanides were synthesised as described in Chapter 2 using the method adapted from Tricot *et al* (1990). Briefly, 1 mole of a diamine of the required length was mixed with 2 moles of methylisourea. The methylisourea was added to ice cold ultra-pure water (UPW) and pH increased to 10 using 5M KOH. The product, when diguanidylated, is not soluble at this pH and will precipitate out of solution. The diamine was added and this was incubated for three hours, performing 15 minute checks to ensure pH was maintained at 10. Precipitates were collected by centrifugation, washed with 10ml ice cold UPW, recentrifuged and this was repeated. Final precipitates collected were frozen at -20^{o}C and freeze dried. MS was carried out by direct injection and MS^2 fragmentation pattern obtained to confirm the required structure had been synthesised. This method has been used to guanidylate the amino acid lysine to facilitate ionisation and therefore observation of lysine containing peptides by matrix assisted laser depolarisation/ ionisation (MALDI) MS (Beardsley and Reilly, 2002).

6.2.5 Synthesis method

Cadaverine is the product of *post mortem* bacterial decarboxylation of the amino acid lysine as shown in Fig 6.16:

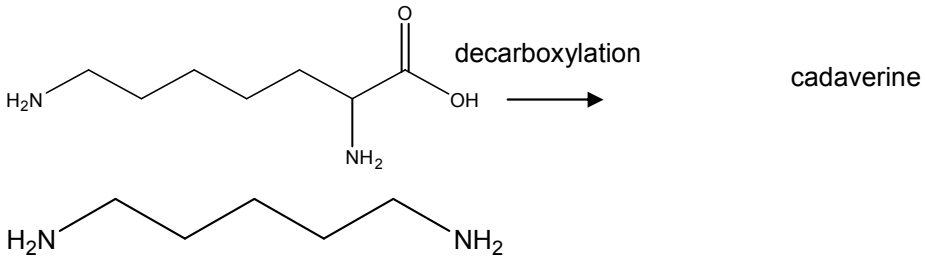


Fig 6.16: Cadaverine is the consequence of *post mortem* decarboxylation of the amino acid lysine in mammalian cadavers.

Using cadaverine as the diamine, it was assumed that DG5 would be generated with a molecular weight of 186. This reaction is shown in Fig 6.17:

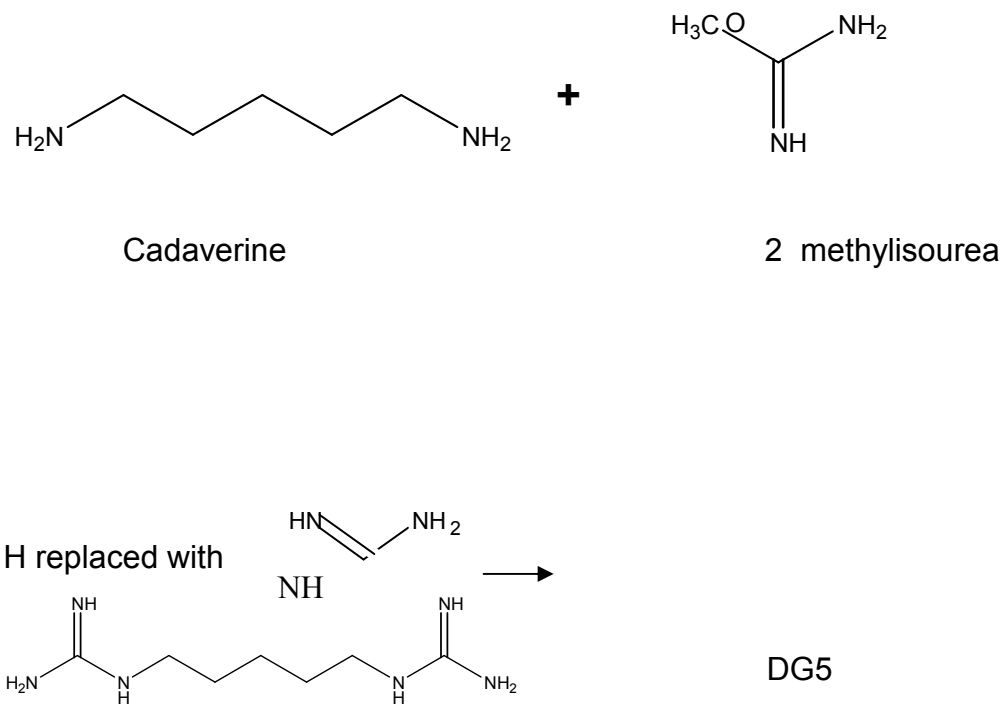


Fig 6.17: Synthesis of DG from cadaverine
Cadaverine, structural formula = $C_5H_{14}N_{2\,=}$ 102amu; DG5, $C_7H_{18}N_{6\,=}$ 186 amu.

The MS data suggested the required compound had been synthesised, as shown in Fig 6.18:

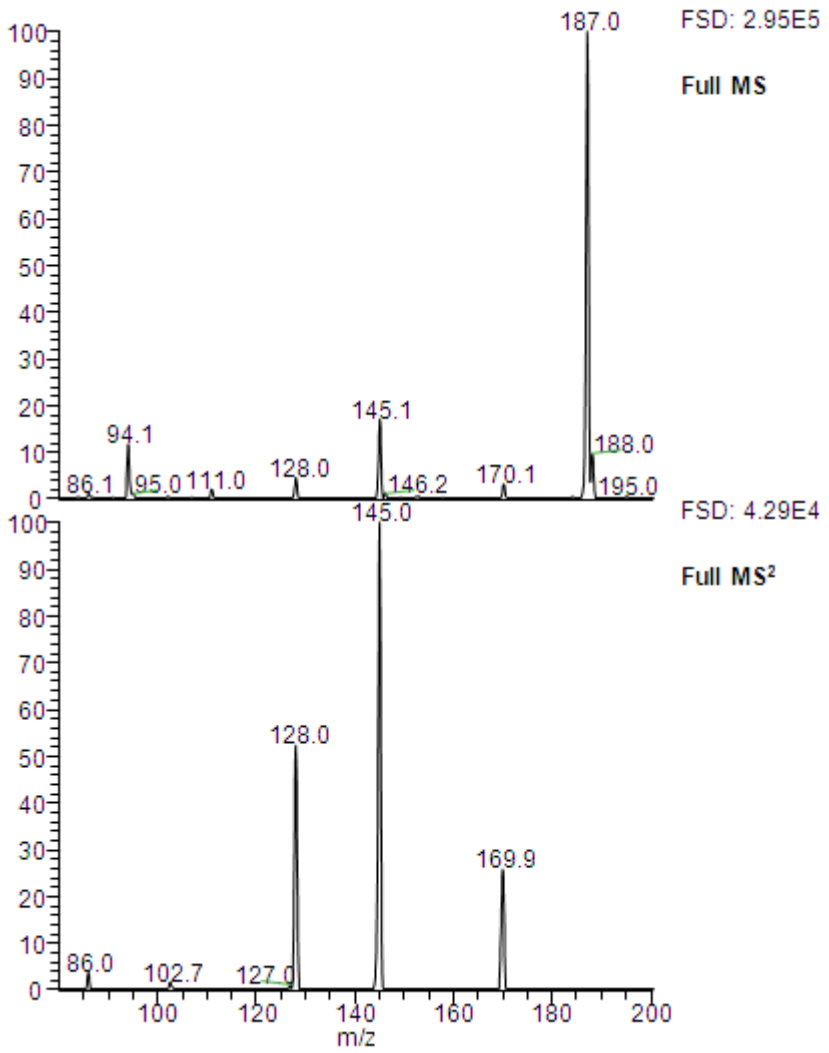


Fig 6.18: Mass spectrometry data for DG5

In positive mode, the full MS data shows a molecule of 186 amu has been synthesized. The MS^2 fragments probably represent the following neutral losses: 187 – 170 = 17 amu = NH_3; 187 – 145 = 42 = CH_2N_2; 187 – 128 = 59 – CH_4N_3; 187 – 86 = 101 – $C_5H_{12}N_2$. The figures in the top right corners are the full scale deflections of the MS detector.

DG7, DG8 and DG12 were then synthesised as shown in table 6.1, simply by replacing cadaverine with the following longer diamines:

Substrate	**Product**
1,7 diaminoheptane	DG7
1,8 diaminoheptane	DG8
Spermine	DG12

Table 6.1: Table showing the substrates and the diguanides synthesised from them.

The MS data suggested the required products had been synthesised, and MS^2 data showed the predicted fragmentation pattern as shown in Figs 6.19:

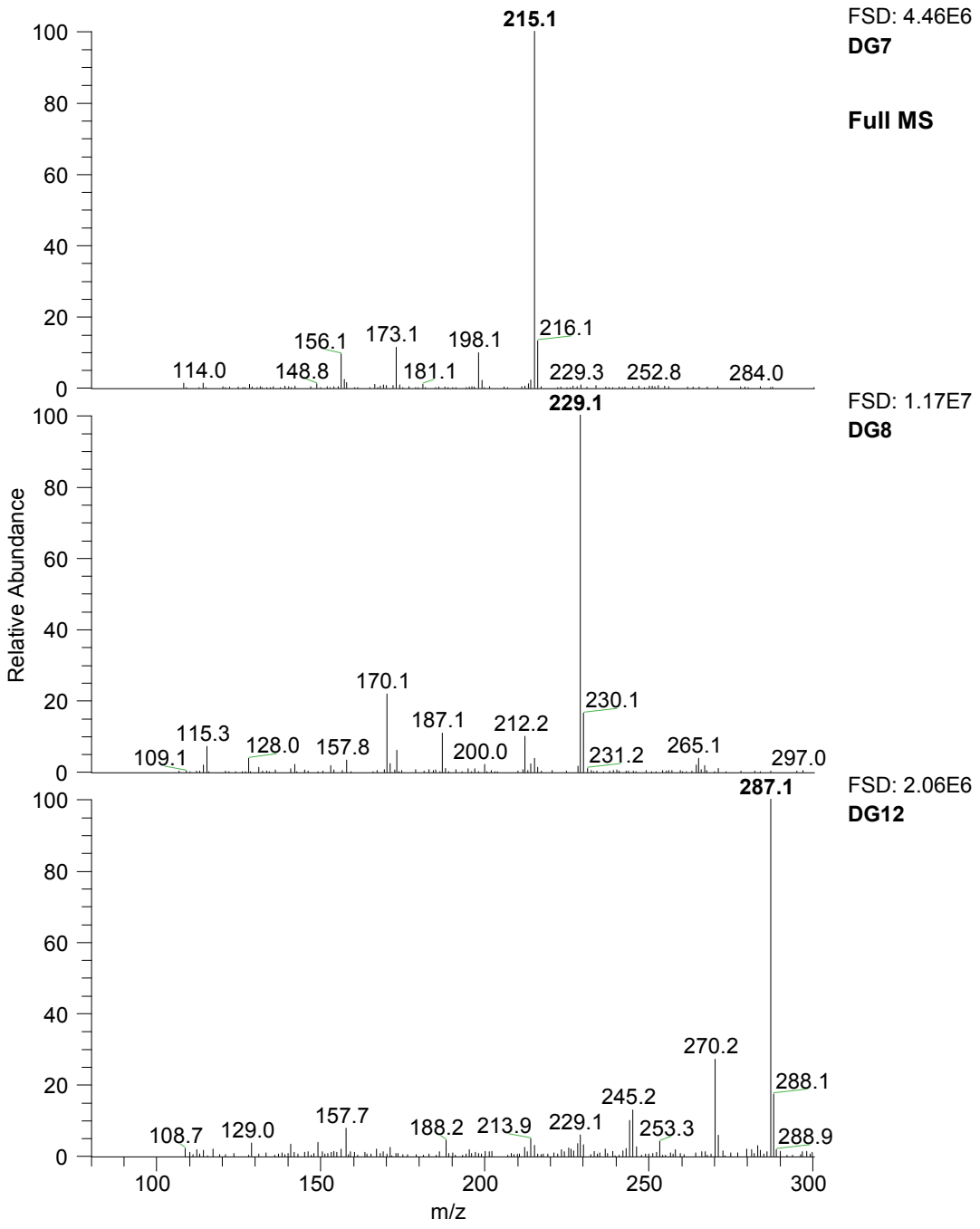


Fig 6.19A: Full mass spectra for DG7, DG8 and DG12.
MS spectra were obtained by direct infusion of samples. Figures in the top right hand corner are the full scale deflections of the MS detector. The main signal is highlighted in bold and other signals may arise from in-source fragmentation of the main peak.

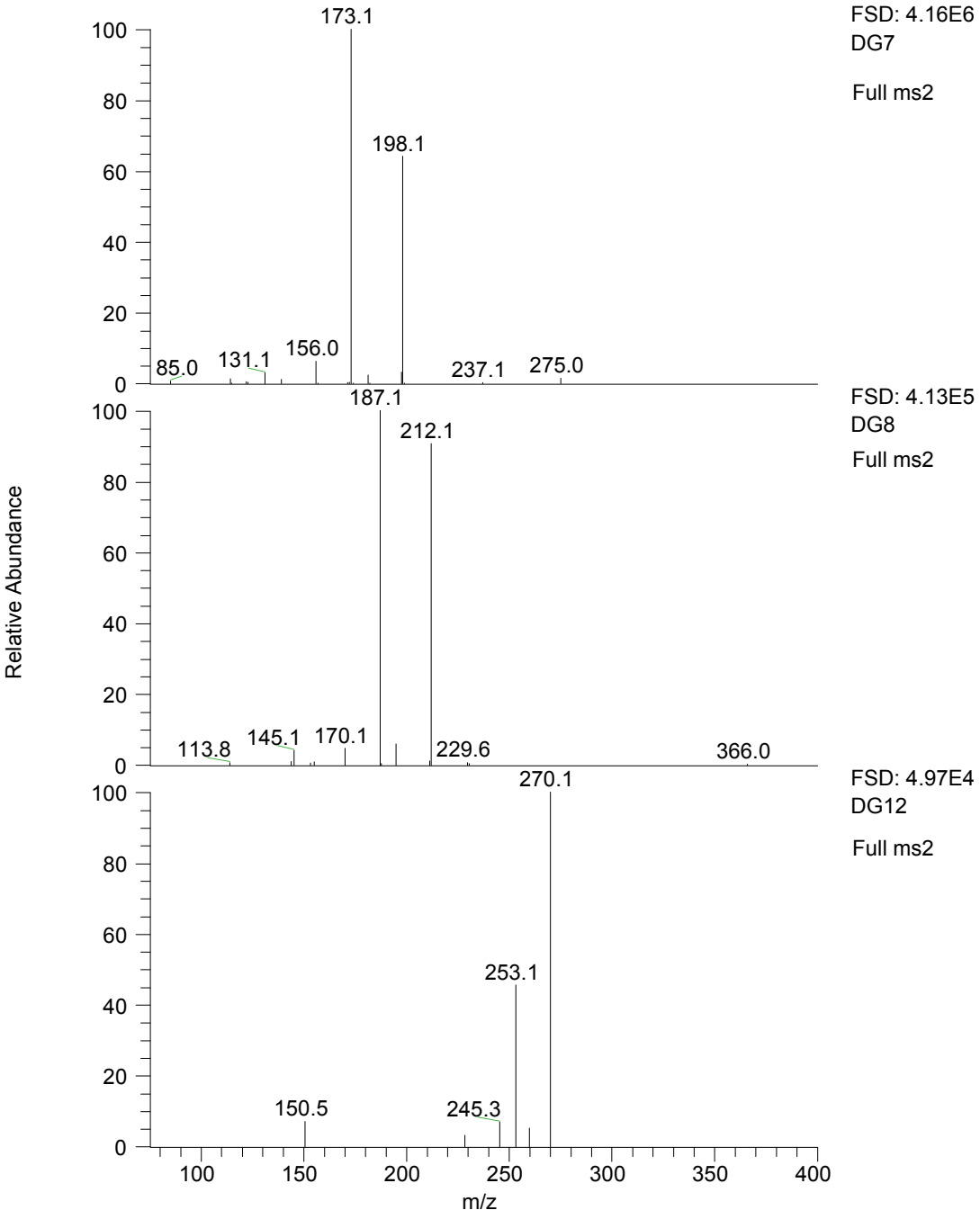


Fig 6.19 B: MS_2 spectra of DG products
Putative IDs:
DG7: 215 – 198 = 17 (NH_3); 215 – 173 = 42 (CH_2N_2).
DG8: 229 - 212 = 17 (NH_3); 229 – 187 = 51 (NH_3 X 3).
DG12: 287 – 270 = 17 (NH_3); 287 – 253 = 34 (NH_3 X 2).
FSDs are shown at the top right of each panel.

6.2.6 Replacement of C4 of DG8 with a secondary amine.

A DG8 analogue was synthesised from spermidine. The structure synthesised (DG8N), is identical to DG8 apart from the C4 being replaced with a secondary amine as shown in Fig 6.20:

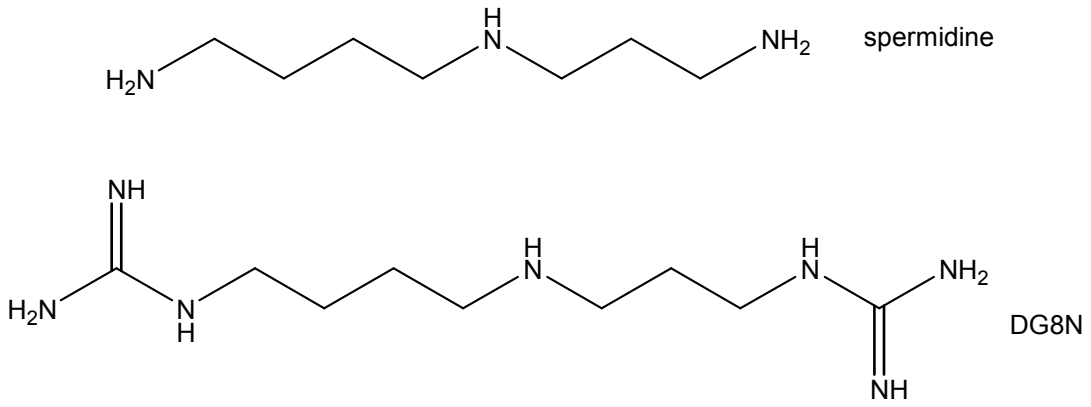


Fig 6.20: Spermidine and DG8N

LCMS analysis was carried out to check that the required compounds had been synthesised and that they were in excess of excess of 95% pure, as shown in Fig 6.21. The purified compounds gave MS^2 fragmentation patterns consistent with DG structures. (Fig. 6.19b).

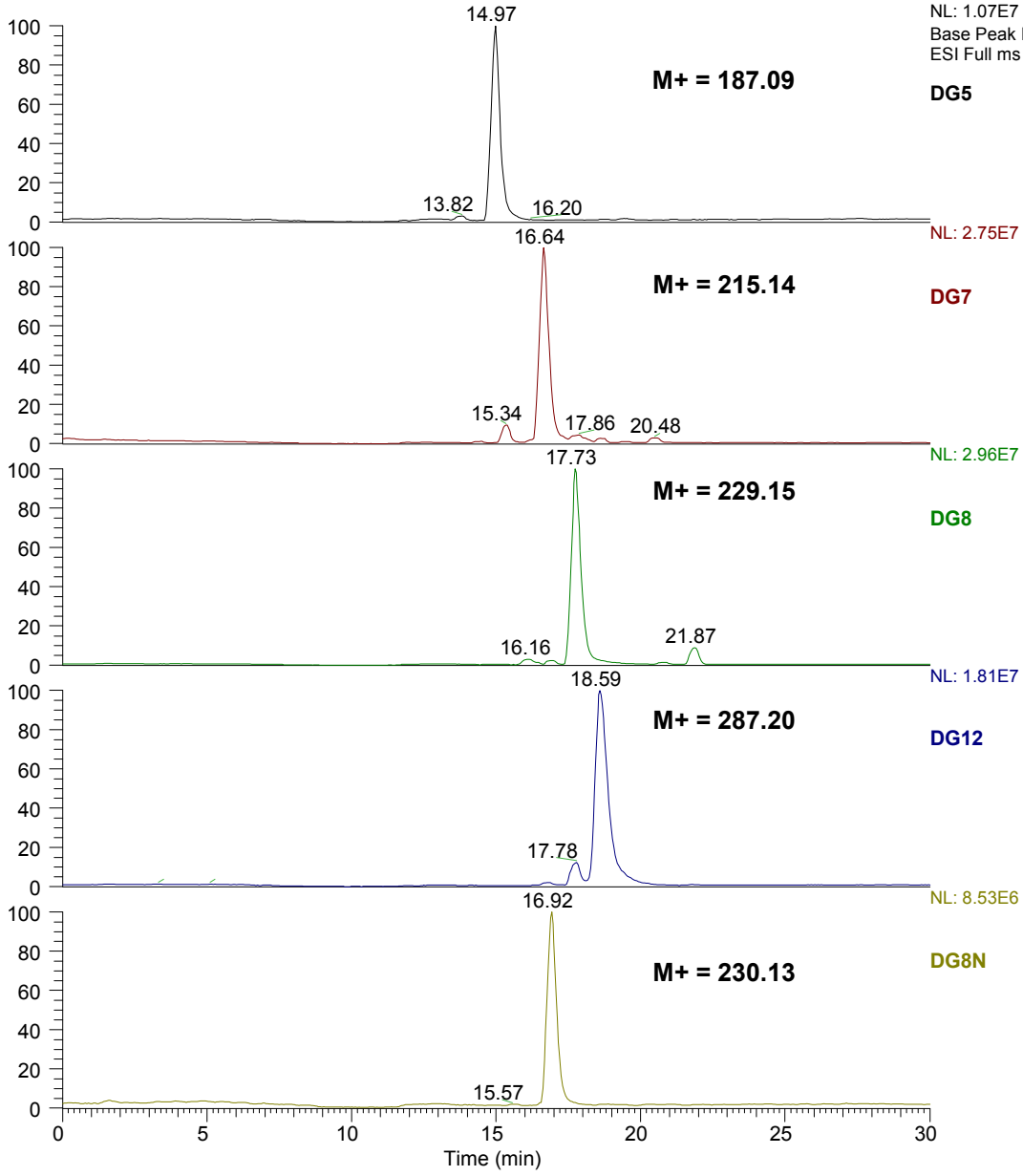


Fig 6.21: LCMS traces of DG5, 7, 8, 12, and DG8N
The MS base peak signal for each sample is shown. Peak area analysis confirms that each sample was in excess of 97% pure. The M+H m/z value for each sample is given in bold. The figures in the top right hand corners are the full scale deflection of the MS detector.

It was found, as chain length progressed, that modifications to the synthesis protocol were required. When no product precipitated from 1,7-diaminoheptane, it was thought that synthesis of a larger molecule may require extra energy therefore the diamine was heated to 37^{o}C before addition of the methylisourea, and a precipitate formed. When a similar problem arose with 1, 8-diaminoheptane its melting point was noted to be 50-52^{o}C, therefore the water bath was heated to this temperature for the duration of the synthesis, again with success. With respect to DG8N, this increase in temperature alone was unsuccessful, but it was found that overnight incubation at 4^{o}C resulted in a successful outcome as the product precipitated out of solution.

6.2.7 Effect of a dose response of DG5 on AMPK, S6 and ACC phosphorylation.

DG5 was applied to cells to determine the effects on cell signalling. Cell lysis and western blotting showed DG5 induced the phosphorylation of AMPK and ACC at 10μM and dephosphorylation of S6 at 100μM. This data is shown in Fig 6.22:

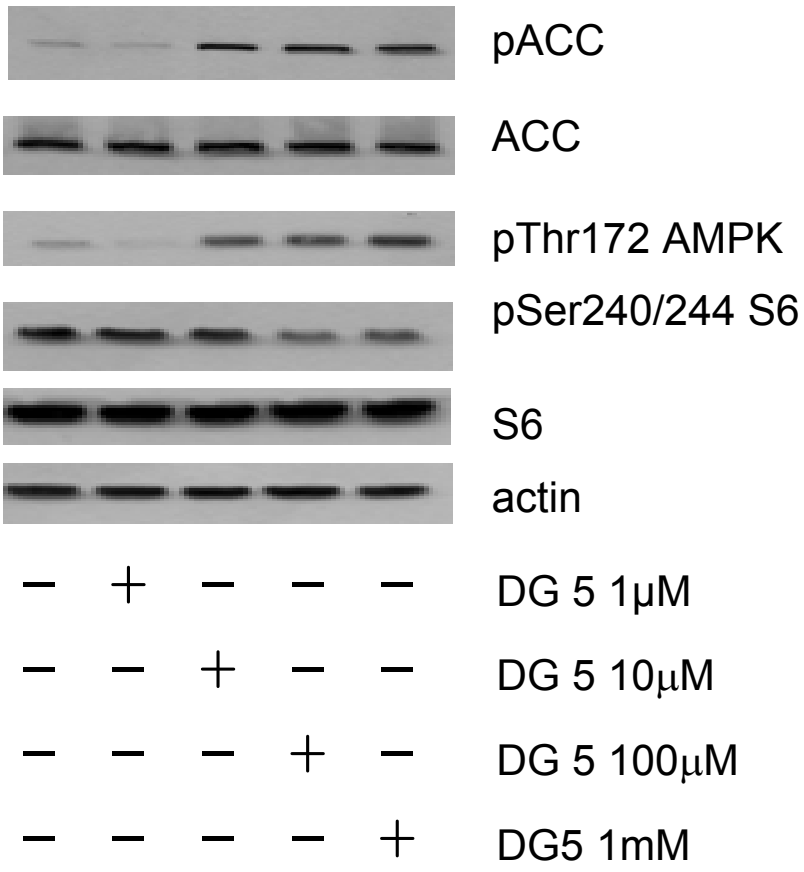


Fig 6.22: Effect of DG5 on phosphorylation of ACC, AMPK and S6
Serum starved H4IIE cells were treated as above for 3hrs followed by lysis and SDS- PAGE as described in chapter 2. Immunoblotting was carried out using 2 ACC antibodies- one which detects total ACC (ACC) and one which detects phosphorylated ACC (pACC), an antibody which detects AMPK only if it is phosphorylated on Thr172 (pThr172 AMPK), 2 S6 antibodies- one which detects total S6 (S6) and one which detects Ser240/244 S6 phosphorylation (pSer240/244 S6) and an actin antibody. This showed DG5 to phosphorylate AMPK and ACC and to dephosphorylate S6 at a concentration of 10μM.

6.2.8 Effect of a dose response of DG7 & DG8 on AMPK, S6 and ACC phosphorylation.

DG7 and DG8 were applied to cells to determine effects on cell signalling. Cell lysis and western blotting showed that DG7 and DG8 induced phosphorylation of AMPK and dephosphorylation of S6 at 3.14μM. This is shown in Fig 6.23:

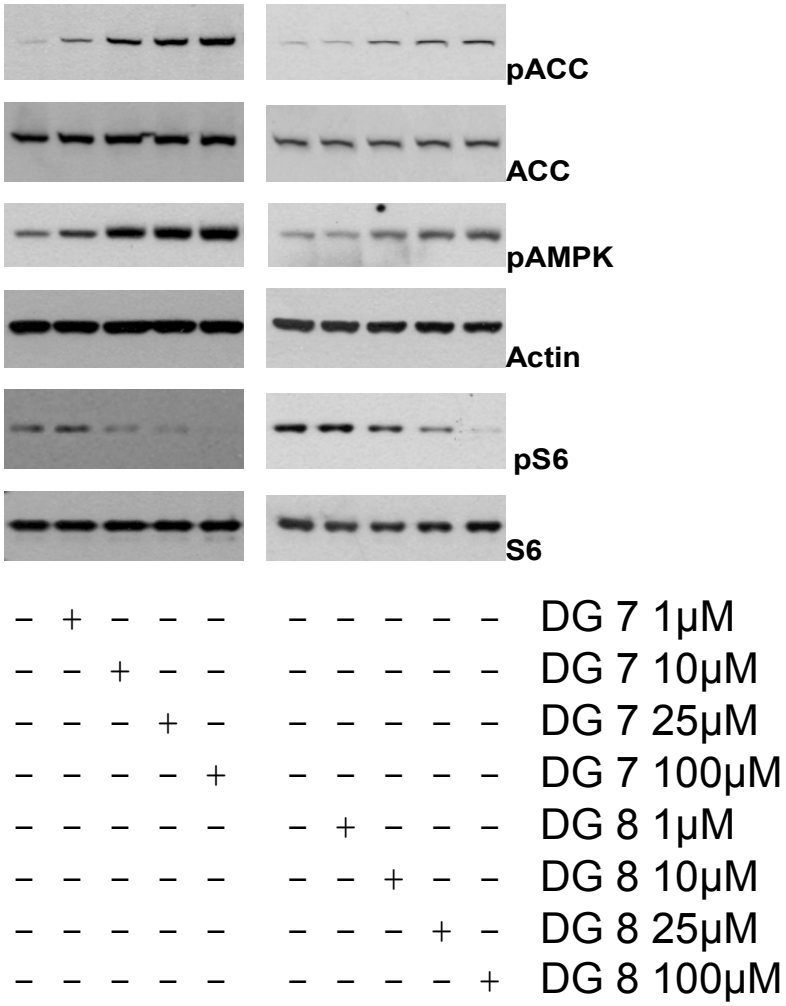


Fig 6.23: Effect of DG7 and DG8 on AMPK, ACC and S6.
Serum starved H4IIE cells were treated as above for 3hrs followed by lysis and SDS- PAGE as described in chapter 2. Immunoblotting was carried out using two ACC antibodies- one which detects total ACC (ACC) and one which detects phosphorylated ACC (pACC), an antibody which detects AMPK only if it is phosphorylated on Thr172 (pThr172 AMPK), two S6 antibodies- one which detects total S6 (S6) and one which detects Ser240/244 S6 phosphorylation (pSer240/244 S6) and an actin antibody.

6.2.9 Comparison of effects of DG8 and DG8N on cell signalling

DG8N and DG8 were applied to cells side-by-side for direct comparison of their effects on cell signalling. Cell lysis and western blotting suggested that although DG8 was effective on AMPK by 5μM and 50μM with respect to ACC and S6, DG8N was ineffective at all concentrations tested. This data is shown in Fig 6.24. Separate experiments suggested that DG8N was ineffective at concentrations into the millimolar range (data not shown).

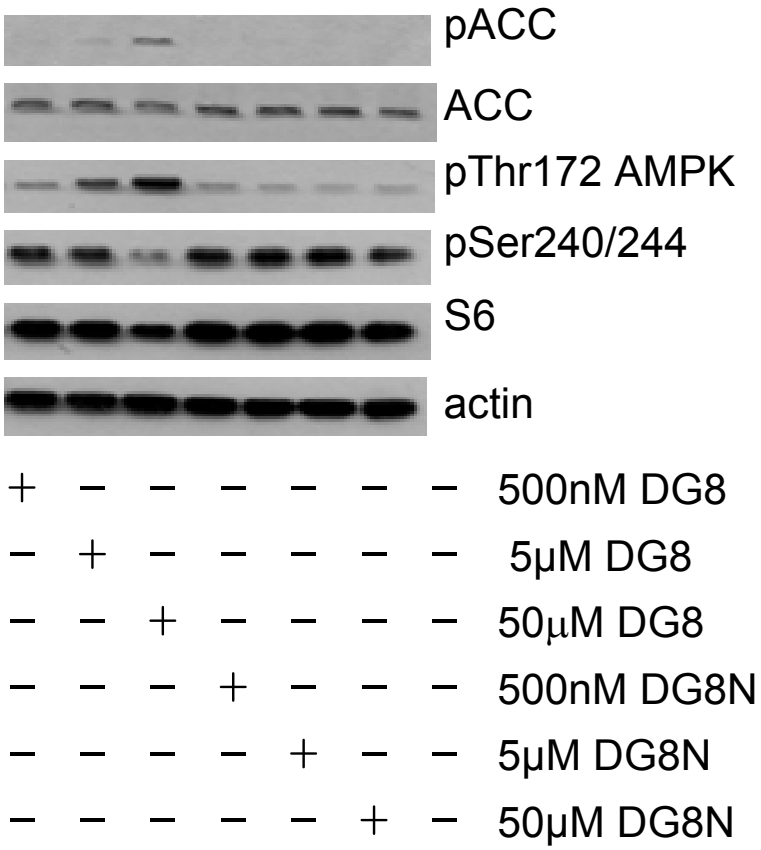


Fig 6.24: Effect of replacement of C4 of DG8 with a secondary amine on cellular responses.
Serum starved H4IIE cells were treated with/without DG8 or DG8N as above for 3hrs followed by lysis and SDS- PAGE as described in chapter 2. Immunoblotting was carried out using two ACC antibodies- one which detects total ACC (ACC) and one which detects phosphorylated ACC (pACC), an antibody which detects AMPK only if it is phosphorylated on Thr172 (pThr172 AMPK), two S6 antibodies- one which detects total S6 (S6) and one which detects Ser240/244 S6 phosphorylation (pSer240/244 S6) and an actin antibody.

6.2.10 Synopsis

When the apparent minimum concentrations of each diguanide required to phosphorylate AMPK were compared, a 'digital' or threshold- type response was observed, in that DG4 was ineffective but DG6-10 affected AMPK, ACC and S6 in the low micromolar range. DG5 alone exhibited efficacy that was intermediate between these two extremes. Interruption of the hydrocarbon chain with a secondary amine abolished cell responses. The same trend was observed with dephosphorylation of S6, except that when a carbon chain length of 10 was reached, potency appeared to reduce.

6.2.11 Glucose 6 phosphatase and cell toxicity

As the accuracy of measurement of AMPK responses is limited by the photodynamic range of the photographic film used, a cell reporter assay to investigate DG responses was carried out. Previous studies showed that metformin represses glucose 6 phosphatase (G6Pase) expression (Foretz *et al*, 2010, Logie *et al*, 2012). Therefore effects of DGs on G6Pase expression were measured using a luciferase reporter assay and IC_{50} concentration calculated as described in chapter 2. These experiments provided further evidence that cellular responses occur at a threshold between DG4 and DG6, as shown in Table 6.2.

Compound	IC_{50} for G6Pase promoter activity (μM)	Cell viability in WST-1 assay IC_{50}, (μM)
DG4	>100	166.90
DG5	15	54.15
DG6	1.6	3.62
DG7	1.7	2.97
DG8	0.75	1.62
DG10	0.5	2.96

Table 6.2: IC50 of DGs on G6Pase-luciferase expression and IC50 on cell viability.
For comparison, the IC_{50} (concentration of drug required for 50% inhibition) for G6Pase in response to metformin was found to be 630μM. Metformin's cell viability IC_{50} measured in the WST-1 assay was >5mM. These experiments were performed by Lisa Logie in the laboratory.

6.3 Discussion

6.3.1 Potency and toxicity of the diguanides on AMPK, S6 phosphorylation and G6Pase expression, increases with hydrocarbon chain length

The potency of the diguanides on AMPK, S6 and G6Pase increased with chain length. It was found that the response was 'digital' rather than 'analogue' in that millimolar concentrations of DG4 were required to phosphorylate AMPK but DG5 to DG10 required a concentration in the low micromolar range to show this effect. The threshold in DG effects described above may be explained if there is a threshold of hydrophobicity that must be reached before these compounds can migrate into the plasma membrane and access the cell. The partition coefficients of each DG and DG8N are shown in Table 6.3:

Diguanide	Log P
DG4	-1.423
DG5	-0.917
DG6	-0.412
DG7	-0.093
DG8	+0.598
DG8N	-1.717
DG10	+1.609
DG12	+2.619

Table 6.3: Hydrophobicity of selected DG compounds. Log hydrophobicity (Log P) was modelled on *molinspiration* software

It can be seen that DG5-DG10 are all more hydrophobic than the inactive compounds DG4 and DG8N, suggesting that a threshold of hydrophobicity is required for these compounds to exhibit efficacy; however, it is important to keep in mind that the compounds might access the cells by alternative mechanisms, for example through drug transporters. Metformin uptake for example, is mediated by the transporter OCT1 (Shu *et al*, 2007). There are other possible explanations for the data. For example, a threshold of hydrophobicity might be required for the drug to enter a hydrophobic pocket on its protein target. The fact that AG12 is equally as active as DG10 indicates that only one guanidine group is required, so the linker is unlikely to be required to separate the two guanidine groups in space.

6.3.2 Possible mechanisms of DGs

Studies on metformin indicate that effects of this drug on both AMPK and G6Pase both depend on inhibition of mitochondrial respiration. The similar threshold exhibited by these two responses in the DG series of compounds

is supportive that AMPK and G6Pase regulation by DGs may also depend on a common mechanism such as mitochondrial inhibition. The ability of diguanides to reduce oxygen consumption is certainly consistent with this possibility (Pressman, 1963). The targets involved may be different however, as metformin is more than an order of magnitude less potent than diguanides and exerts effects on G6Pase without cellular toxicity.

These results are similar to results on glycemia obtained in animal studies in the 1920s with these compounds, although these investigators did observe a slightly more graduated response. For example, they found that the hypoglycemic effect of DG5 was only five fold greater than DG4 and that DG6 was ten-fold more potent than DG5 and equally as potent as DG10 (Kumagai *et al*, 1928). Taken together though, these studies suggest that in this series of compounds, effects either on AMPK activation, S6 inhibition or G6Pase repression are probably equally useful cellular markers of antihyperglycaemic activity.

6.3.3 *Conclusions*

6.3.3.1 *Comparison of biguanides and diguanides*

A comparison of biguanides and diguanides was carried out. A variety of diguanides of incrementally increasing chain length were synthesised and purity confirmed using LCMS. In cell based assays, like the biguanides, diguanides induce AMPK phosphorylation, suppress S6 phosphorylation and repress G6Pase expression. Although diguanides of a chain length of six or greater were most effective, these compounds were also the most toxic as both efficacy and toxicity increased markedly above a threshold of

hydrophobicity. DG5 was the only DG whose toxicity was appreciably lower than the concentration required to phosphorylate AMPK and that of the IC_{50} for G6Pase. These narrow therapeutic windows in cells contrast sharply with metformin and probably explain the lack of clinical utility of these DGs.

CHAPTER 7- CONCLUSIONS

7.1 Introduction

This project has highlighted several groups of compounds and individual compounds which have insulin-like properties in cell culture experiments, in that they induce the phosphorylation of and inactivation of the transcription factor FOXO1a. We also studied agents with metformin-like properties, which activate AMPK signalling and reduce phosphorylation of the ribosomal protein S6. Both these effects are believed ultimately to result in downregulation of gluconeogenesis. Additional drugs capable of restoring suppression of gluconeogenesis in T2D might be useful therapeutically.

Drugs usually act on cells by 'lock and key' interaction with specific molecular targets but various factors have been shown to influence efficacy and toxicity, both at a cellular level and in the whole organism. These properties contribute to what makes a good drug. They include hydrophobicity (Lipinski *et al*, 2001), molecular size (Lipinski *et al*, 2001) flexibility (Veber *et al*, 2002), hydrogen bonding characteristics (Lipinski *et al*, 2001), transport properties and metabolic stability. Besides simply screening for compounds regulating AMPK and FOXO1a, the relationship of a few of these properties to drug action have been investigated.

7.2 Plant extracts with insulin or metformin- like cell signalling properties

When screening plant extracts, it was noted from MS data that proanthocyanidin (PAC) rich fractions were frequently those which induced phosphorylation of FOXO1a, AMPK and reduced phosphorylation of S6. We are unable to conclude that the active agents were PACs themselves

because we were unable to obtain pure preparations of the PACs. Despite this difficulty, we were able to make the following observations:

(i) Fractions containing B-type PACs were more effective inducers of FOXO1a phosphorylation than fractions containing A-type PACs, suggesting that if PACs are the active agents, B-type linkages may be required.

(ii) Effects of PAC-rich fractions did not correlate closely with antioxidant properties of the fractions

(iii) In GSE, which induced phosphorylation of FOXO1a and AMPK, there were more gallic acid and galloylated molecules than in PBE, which was much less active in our cell assays.

7.3 Gallic acid analogues

To circumvent the difficulties that we encountered when attempting to purify compounds with insulin or metformin-like activity from plant extracts, we adopted a candidate-approach that was partly based on the observations made during the fractionation work, where a higher proportion of galloylated molecules was detected in GSE than in PBE. As GSE was also more active on FOXO1a and AMPK, it was decided to assay purified gallic acid itself for these effects on H42E cells. This resulted in a positive response on AMPK signalling. A variety of commercially available hydroxybenzoic acid (HBA) analogues of the GA structure were investigated for similar effects. This work found that GA and another HBA, salicylate, which has known antihyperglycaemic properties, were the only two compounds active on AMPK signalling. Structurally similar to the mitochondrial uncoupler 2, 4-

dinitrophenol, GA and salicylate are themselves uncouplers and the studies in this thesis are consistent with the possibility that this contributes to the effects on AMPK that were observed. A variety of other analogues were tested but further work will be required to determine the mechanisms underlying the structure/activity relationships observed.

7.4 Diguanides

With respect to phosphorylation of AMPK by diguanides, a chain length of 6 carbons or more appeared to be required for maximal response, which probably reflects a hydrophobicity threshold required for efficacy. In many respects the responses to diguanides and biguanides were similar but the clinical experience is that diguanides are far more toxic in clinical use. We observed a similar difference in toxicity at the cellular level. It may be that diguanides are less specific than biguanides or alternatively, biguanides and diguanides might exploit different targets in the mitochondria altogether. Further work to explain this difference in toxicity might therefore enable better illumination of metformin's mechanism of action.

7.5 Discussion

As the compounds and extracts that have been studied are capable of inducing insulin like effects on the IIS pathway and/ or biguanide like effects on AMPK, confirmation of their mechanism of action may lead to the development of additional pharmacological interventions for the treatment of the insulin insensitivity of type 2 diabetes. If unsuitable for clinical use, whether due to toxicity or lack of specificity, it may be possible to modify

these structures in such a way as to overcome these problems, or to identify other molecules with similar targets and properties.

Despite the move to synthetic compound libraries in recent decades, plant-derived compounds continue to be investigated for preventive and/or therapeutic properties in cancer (McDougall *et al*, 2008a), cardiovascular disease (Nigdikar *et al*, 1998), Alzheimer's disease (Ferruzzi *et al*, 2009) and type 2 diabetes (Cameron *et al*, 2008). Type 2 diabetes has a particularly promising window of opportunity for treatment because it often precedes these other life threatening conditions which could possibly be prevented by more effective treatment of type 2 diabetes. For treatment of this condition, studies on plants have already contributed to the discovery of metformin and salicylates. The former is the most widely prescribed type 2 diabetes medicine in the world and the latter is not used clinically but has enjoyed renewed interest in recent years. To help reverse the unfolding epidemic of this disease, our studies on analogues of these drugs and other plant–derived compounds suggest that it may be possible to identify further treatments for type 2 diabetes based on plant products.

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